Science and Technology Facilities Council

Basics of Cryogenic Electron Microscopy

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February 2024
SEA-COAST2024

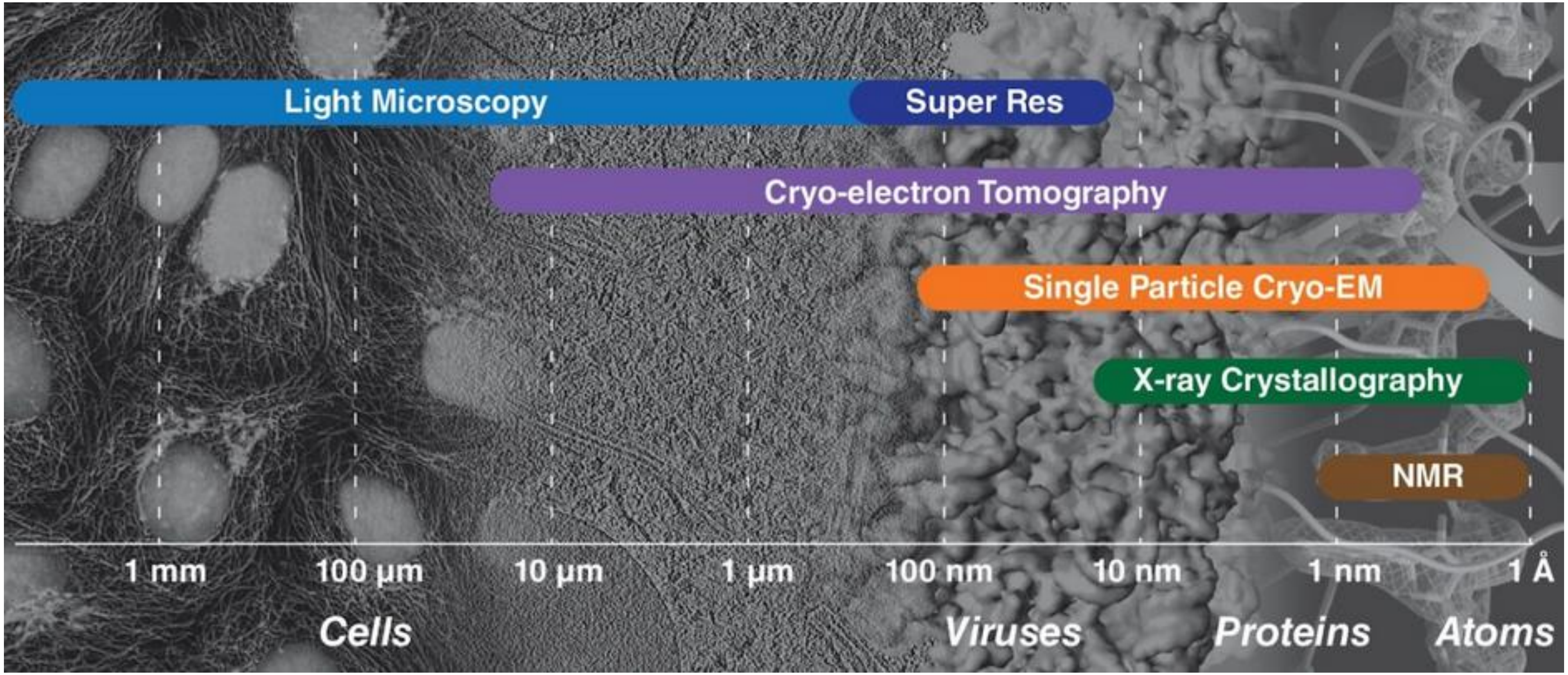
CCP-EM

Research Complex at Harwell

Science and Technology Facilities Council

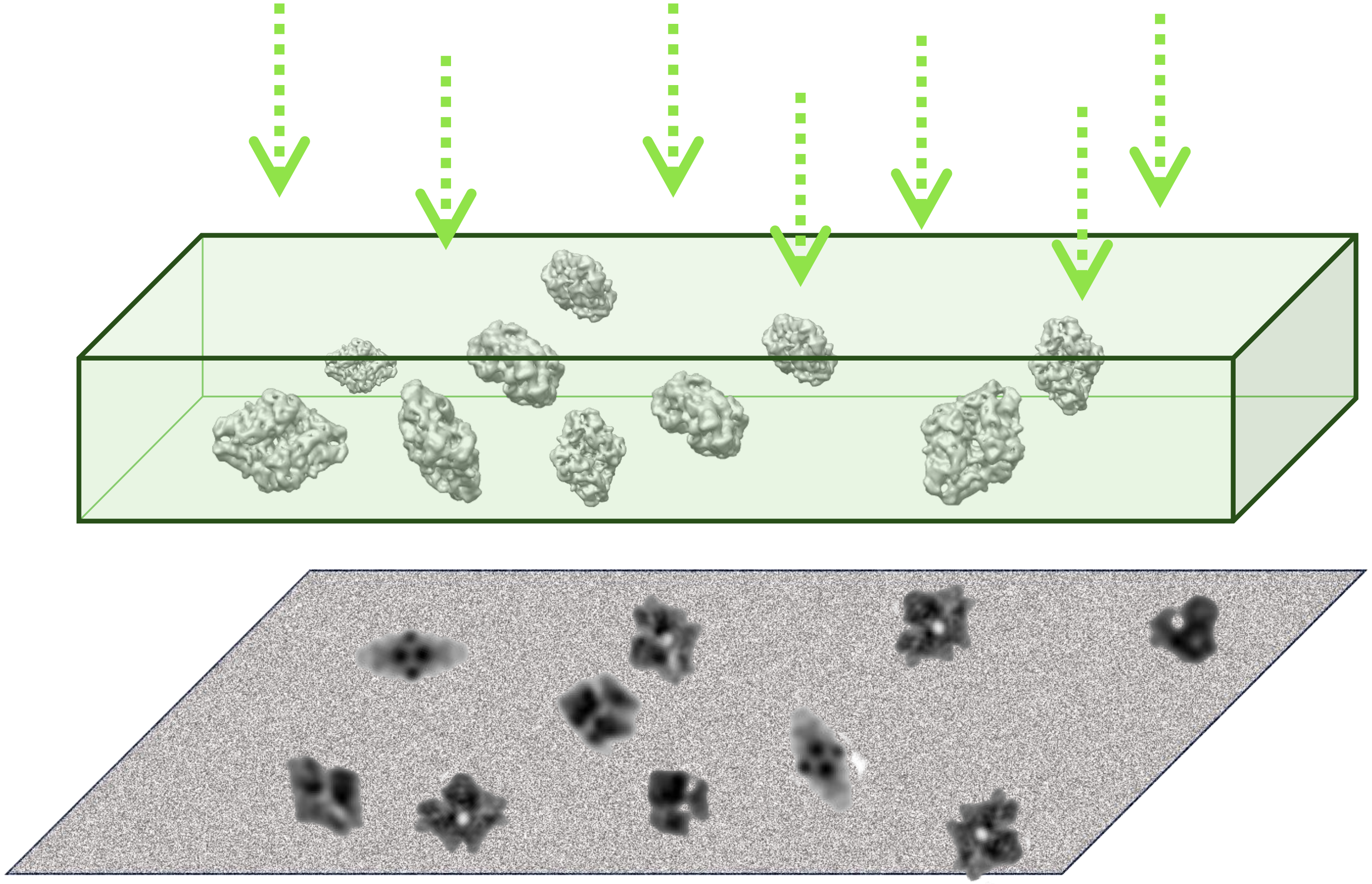
CryoEM in Structural Biology

Biological lengthscales vs techniques



The gap between microscopists and crystallographers is being closed ….

CryoEM imaging (simplistic)



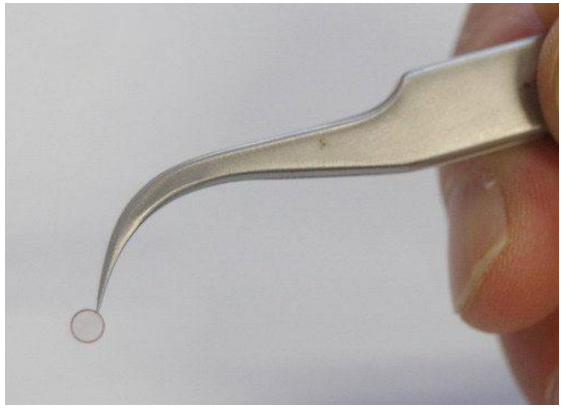
Electron beam

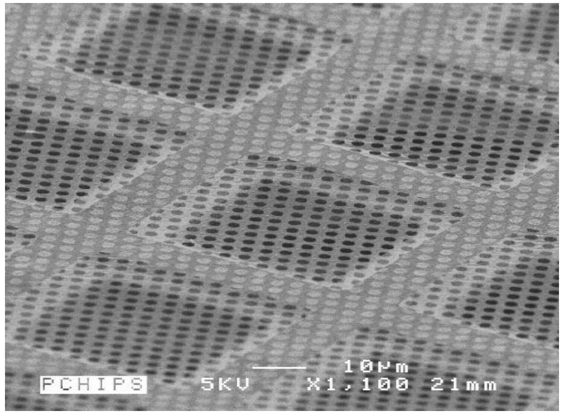
Macromolecules suspended in vitreous ice

2D projection images

Need to recover 3D information → 2 techniques

Single Particle Analysis (SPA)

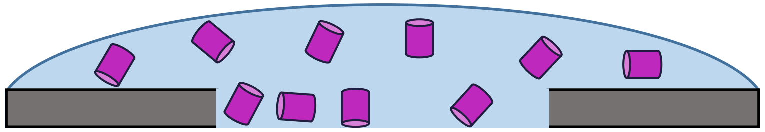




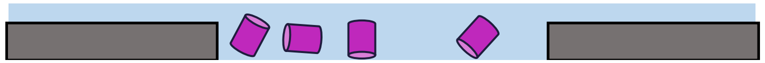
(Quantifoil inc.)

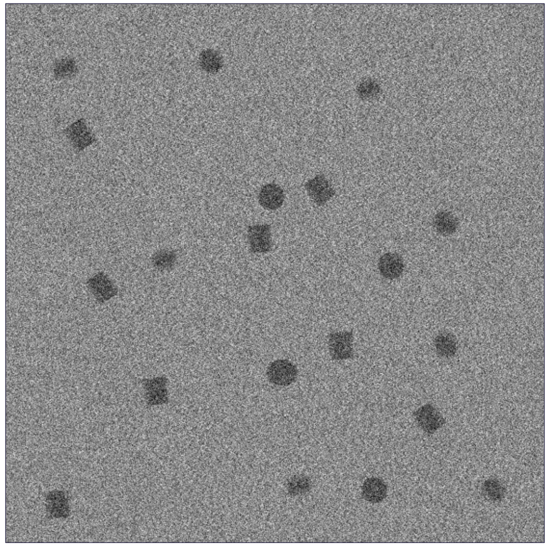
Empty carbon hole

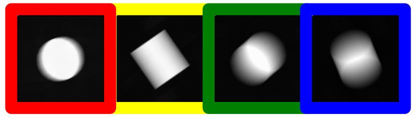
Apply sample

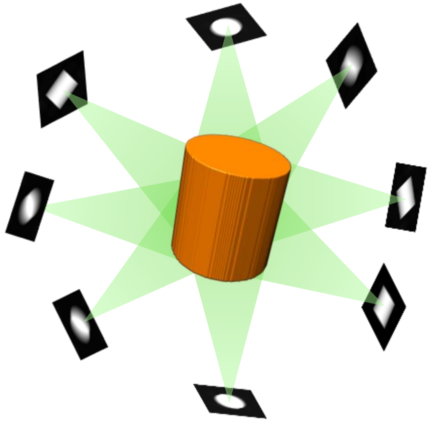


Blot and vitrify



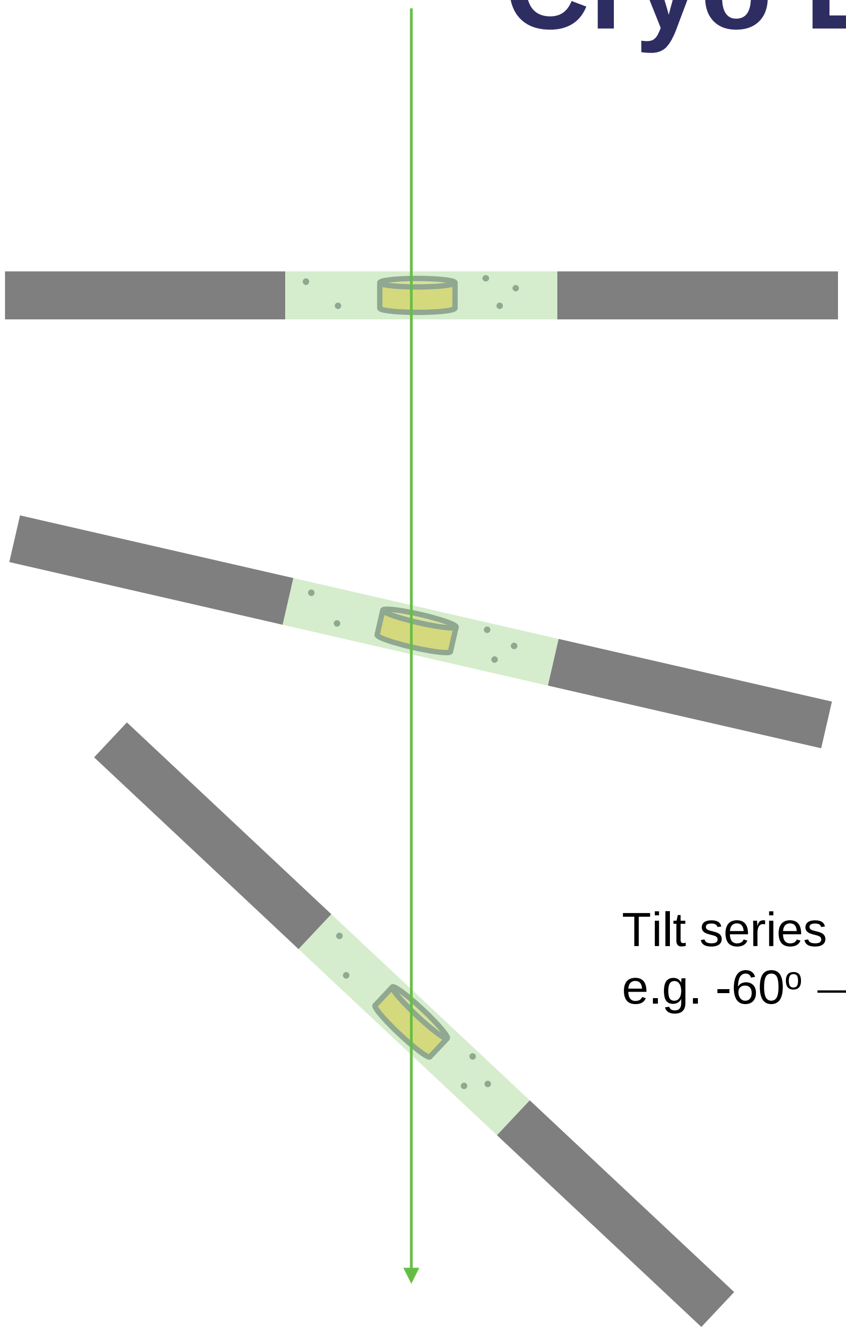




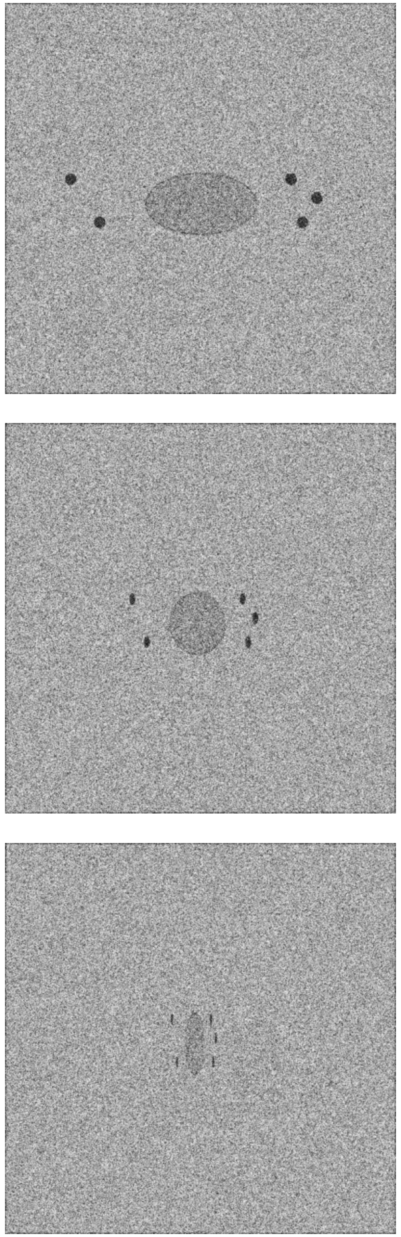


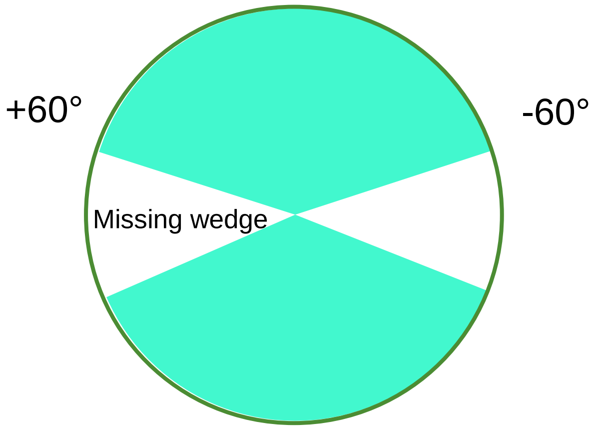
Combine views of identical particles

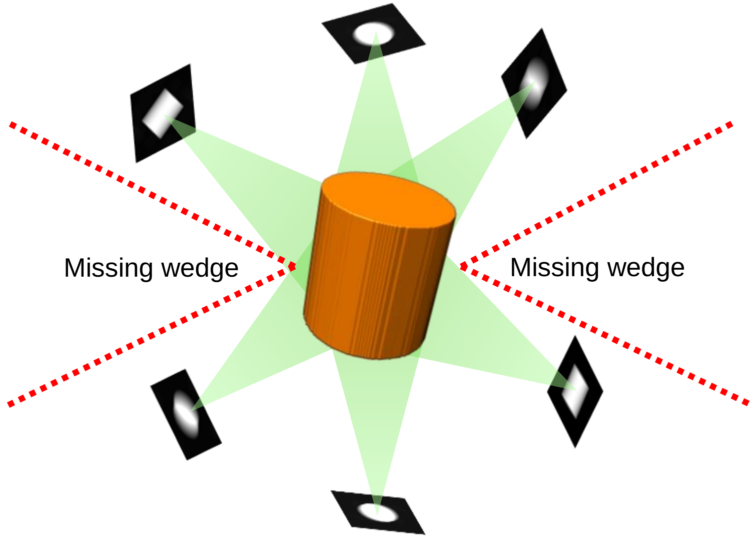
Cryo Electron Tomography



Tilt series
e.g. -60° → +60°





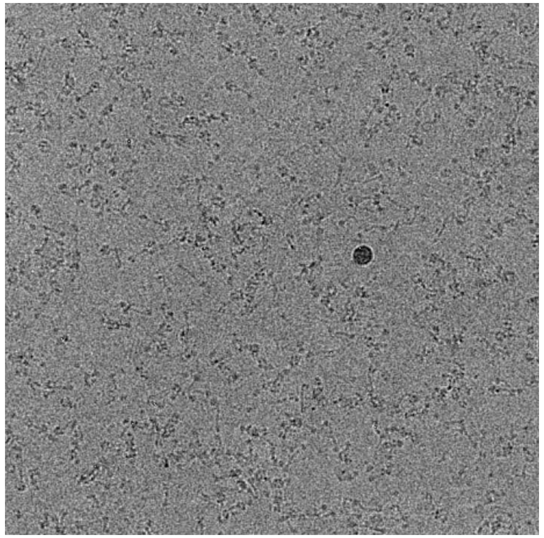


Combine multiple views of same particle

Applications and Limitations of SPA

Applications:

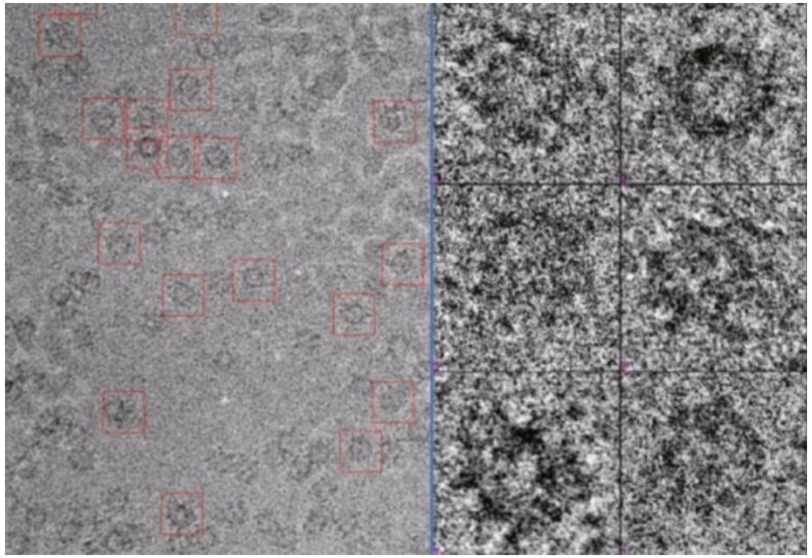
- Proteins that do not crystallize well
- Large macromolecular complexes (ribosomes, viruses)
- Membrane proteins
- Helical filaments (amyloid fibrils, helical viruses)



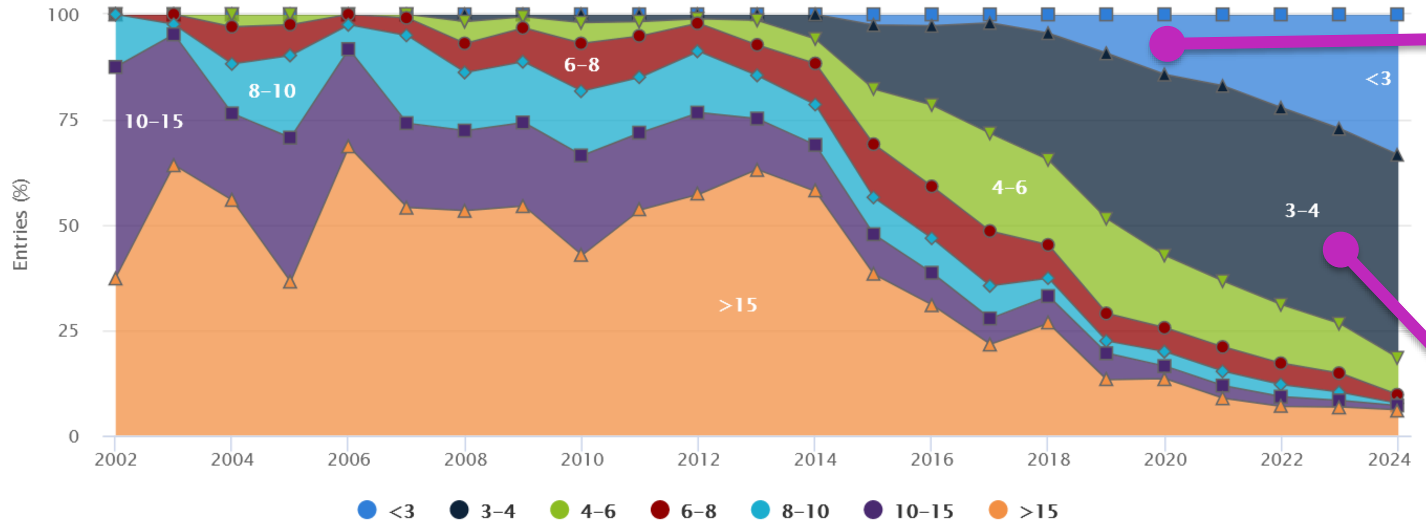
More realistic micrographs

Limitations:

- Requires pure protein sample
- Requires optimization of grid
- Assumes many views of identical particles, good spread of orientations
- Becomes increasingly difficult with smaller proteins
- Need to maintain low radiation dose

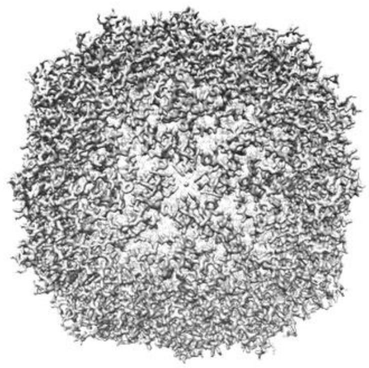


Achievable resolutions



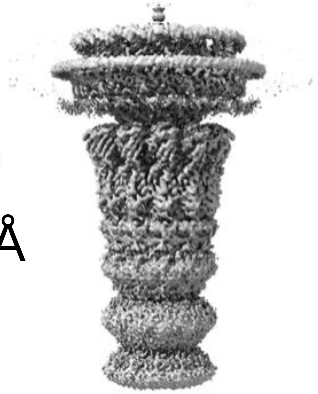
2002 2014 2024

EMD-11668



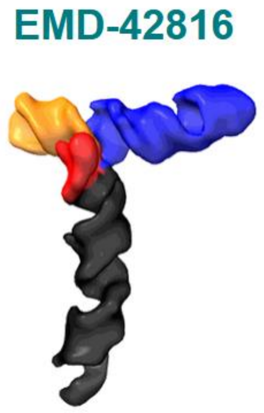
Human apoferritin at 1.15Å
All 19 better than 1.5Å are apoferritin – it is a rock!

EMD-35006

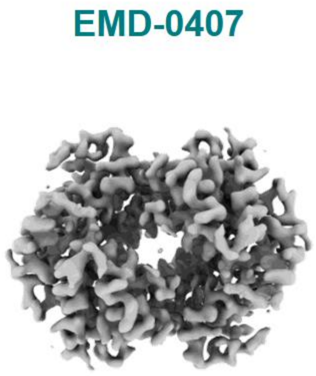


Neck of the DT57C bacteriophage at 3.4Å
More typical.
No fitted model.

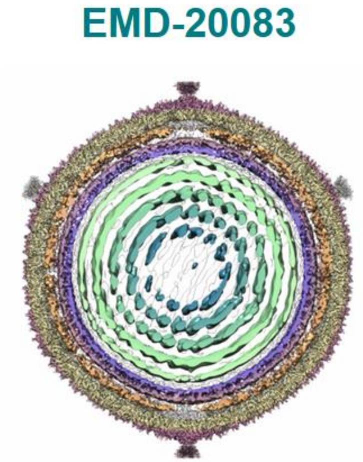
CryoEM structures highly varied



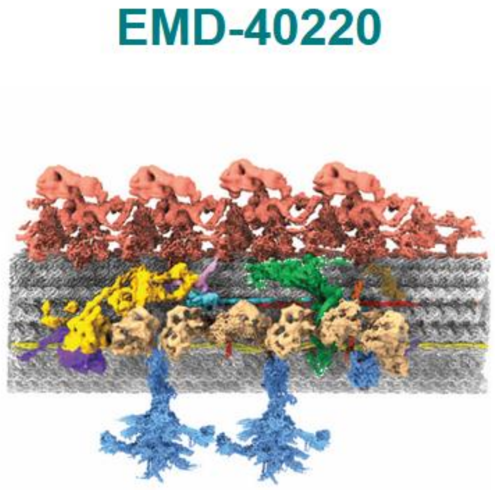
RNA stem-loop
7.1Å, 45 kDa



Methemoglobin
2.8Å, 61 kDa

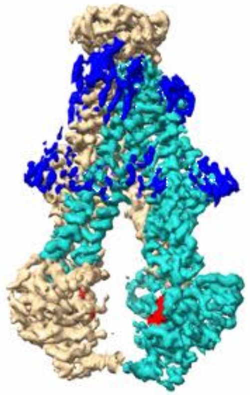


SPV1 (virus)
3.7Å



Doublet microtubule
3.1Å

ABC transporter
BmrCD 3.27Å



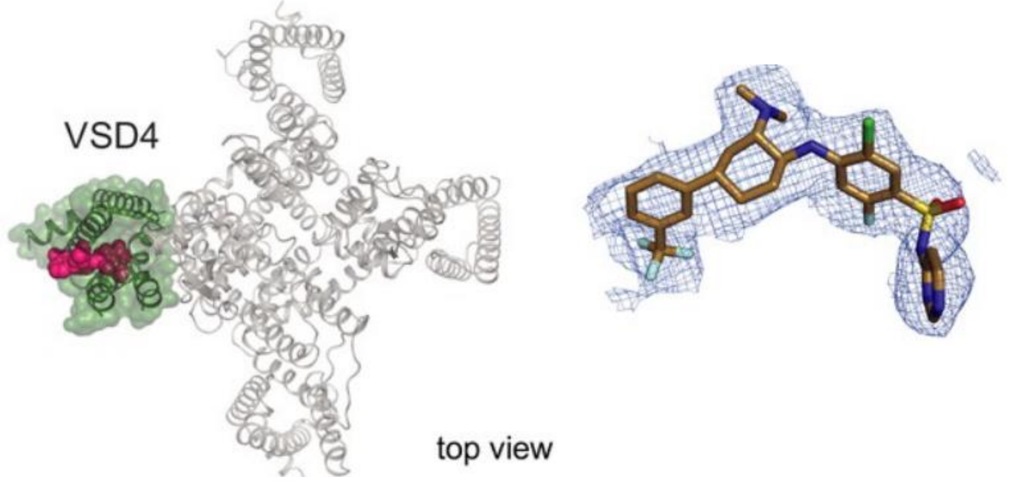
EMDB @ 6th February 2024
32,701 released entries

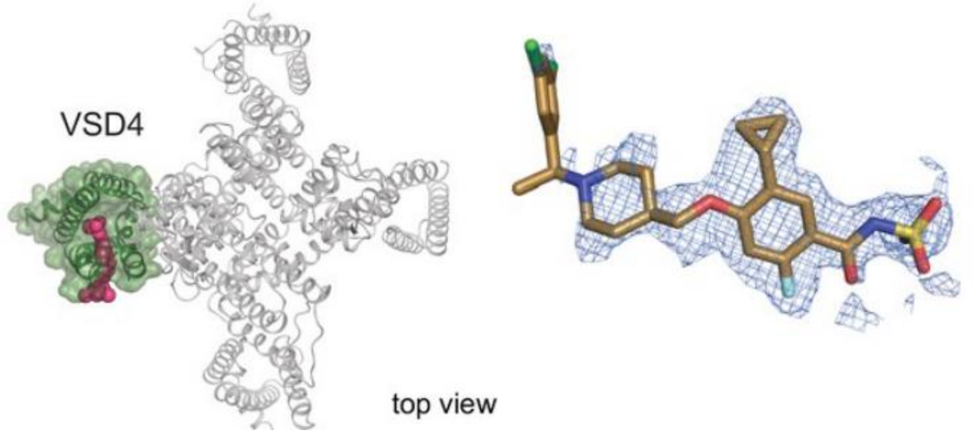
https://www.ebi.ac.uk/emdb/

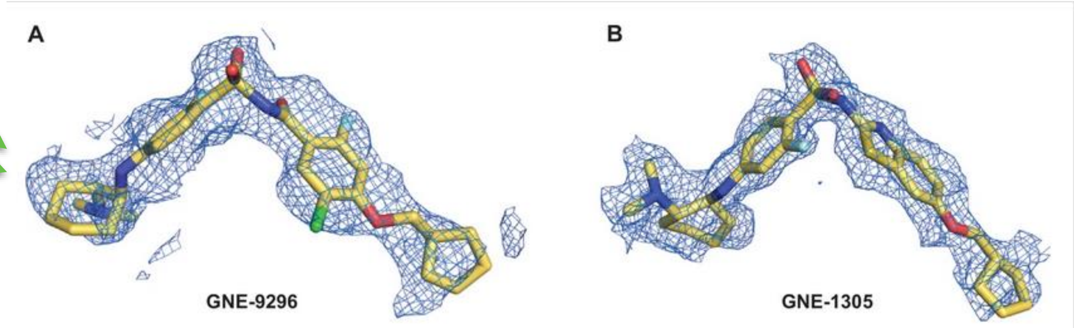
Viewing ligands

Structure-based design of novel hybrid inhibitors targeting a human sodium channel which is a pain target.
Kschonsak et al. *eLife* (2023)

EMD-28778/8F0R	VSD4-NaV1.7-NaVPas channel chimera bound to the arylsulfonamide inhibitor GNE-3565	2.9Å
EMD-28777/8F0Q	VSD4-NaV1.7-NaVPas channel chimera bound to the acylsulfonamide inhibitor GDC-0310	2.5Å
EMD-28779/8F0S	VSD4-NaV1.7-NaVPas channel chimera bound to the hybrid inhibitor GNE-9296	3.1Å
EMD-28776/8F0P	VSD4-NaV1.7-NaVPas channel chimera bound to the hybrid inhibitor GNE-1305	2.2Å



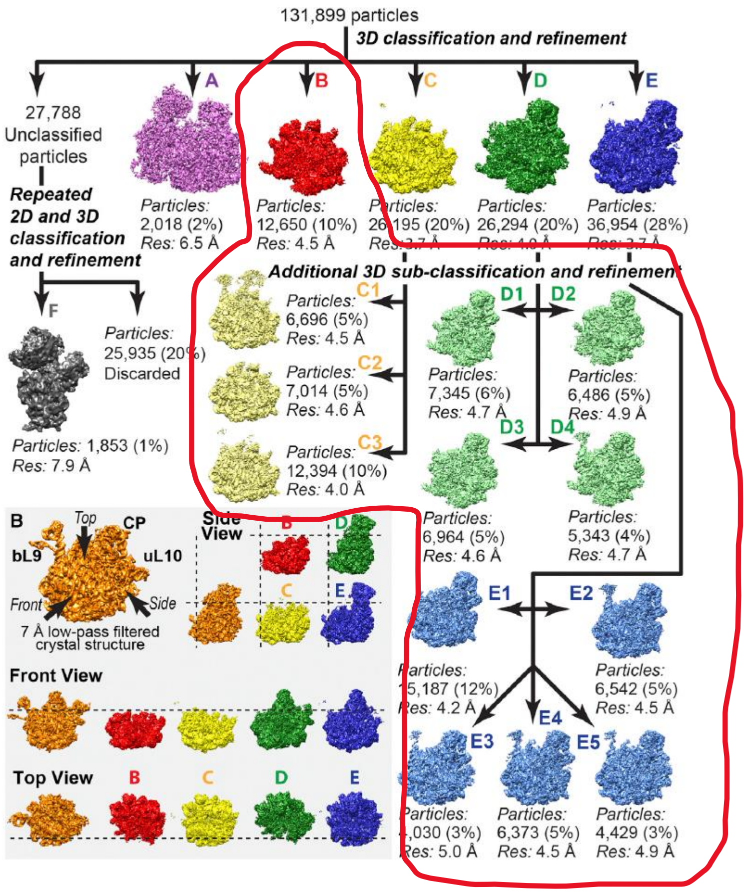




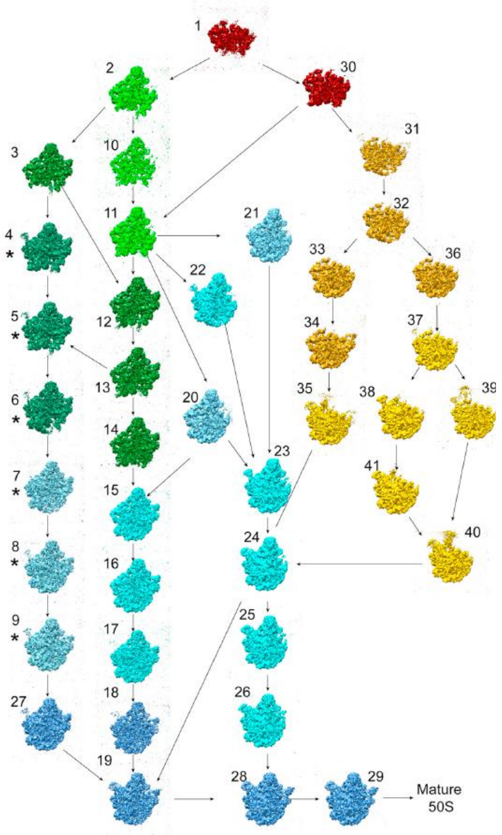
Hard to express full length human, so human cockroach hybrid. Voltage-sensor domain 4 humanised.

Identifying structural classes

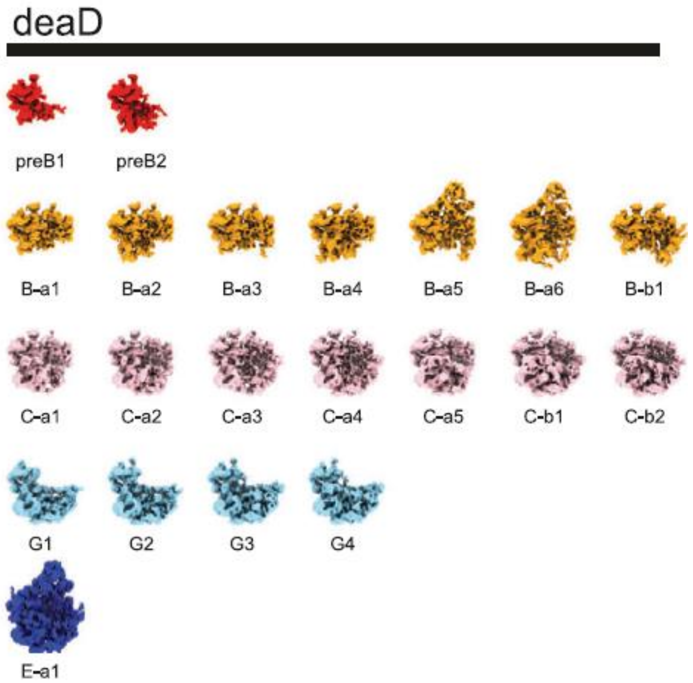
Assembly intermediates of E.coli ribosome LSU



Davis et al Cell (2016)
bL17 depletion, 13 classes



Rabuck-Gibbons et al (2022)
bL17 depletion, improved analysis, 42 classes



Sheng et al *Nat Comm* (2023)
ΔdeaD, earlier intermediates,
21 classes

In situ studies

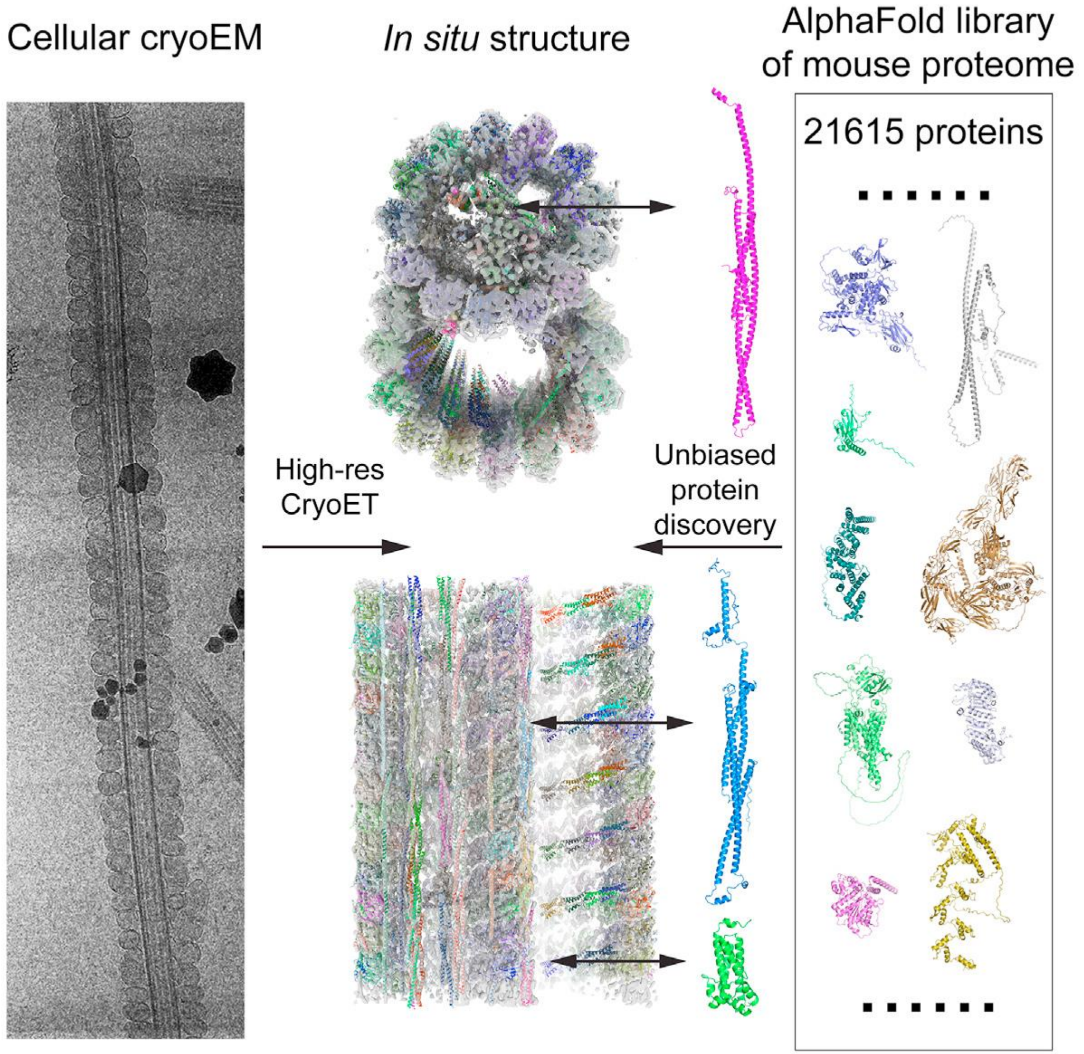
Limits of studying isolated complexes:

- Complex unstable in purification
- Incorrect local environment e.g. lipid composition, electrochemical gradients
- Cannot investigate dependence on physiological state of cell
- Cannot study spatial organisation within cells / organelles

=> in situ cryoET studies

In situ does not always mean tomography:

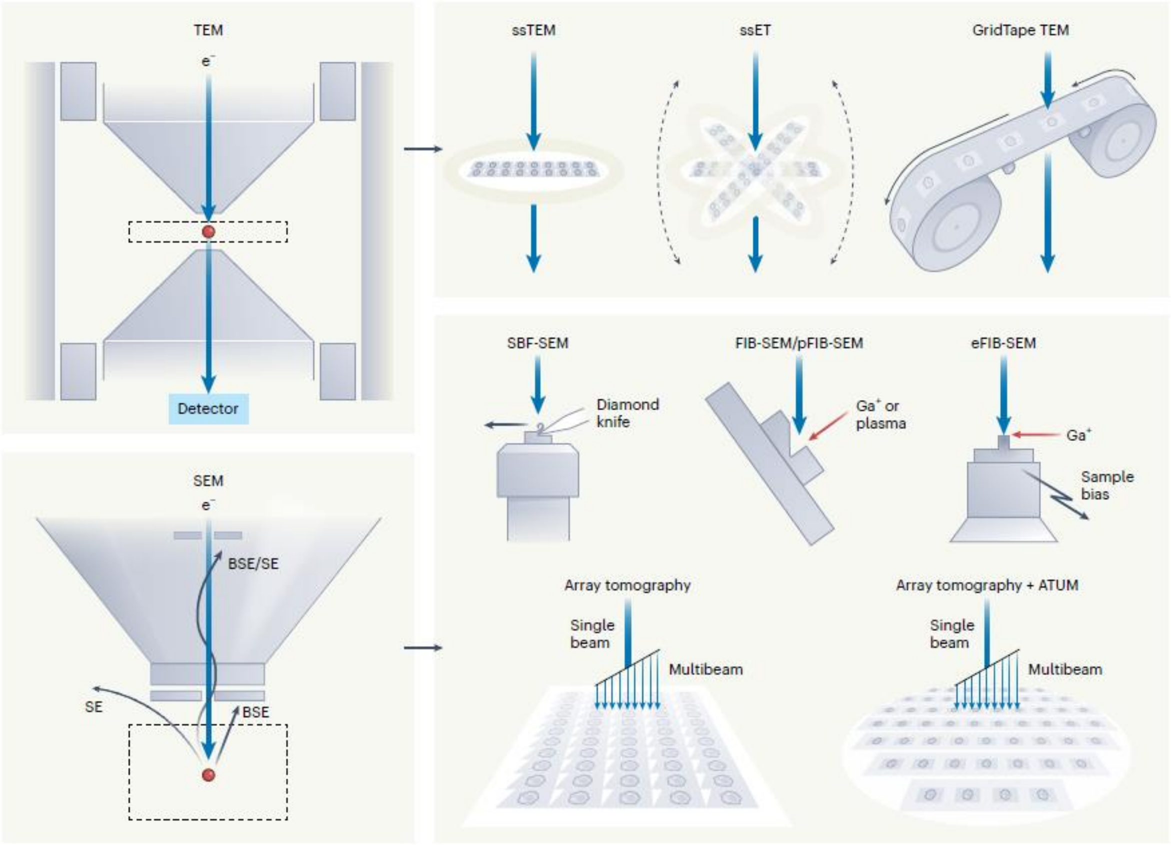
- ➢ isSPA method
  (Cheng et al., 2023, 2021)
- ➢ Baited reconstruction
  (Lucas et al. bioRxiv 2023)

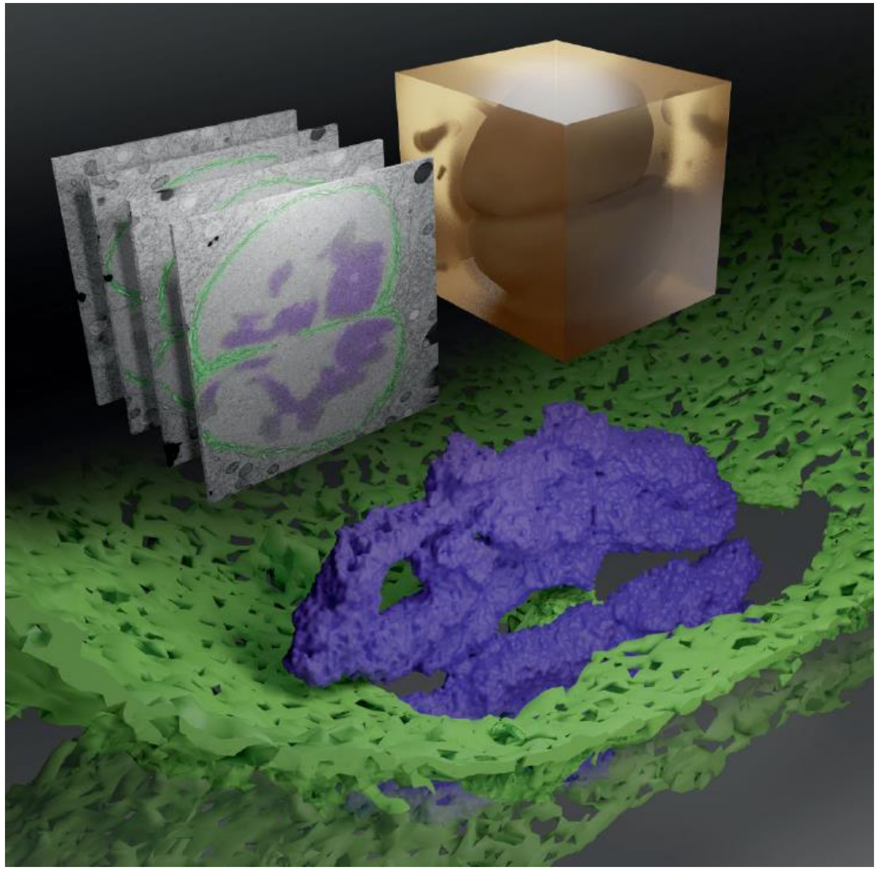


CryoET of microtubule doublets in mammalian sperm.
Sub-tomogram averaging to 6Å.
In situ necessary to avoid loss of components.
Chen et al. *Cell* (2023)

volumeEM

A group of techniques that reveal the 3D ultrastructure of large cell or tissue volumes at (relatively) high resolution.





High-pressure frozen, stained and resin-embedded *C. elegans* zygote (amber block 22 × 14 × 16 µm). Imaged by FIB-SEM to generate 9 × 9 × 9 nm voxel image volumes.

Collinson et al. (2023) “Volume EM: a quiet revolution takes shape” *Nature Methods* **20**, 777

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Fundamentals of cryoEM

Electron microscopes

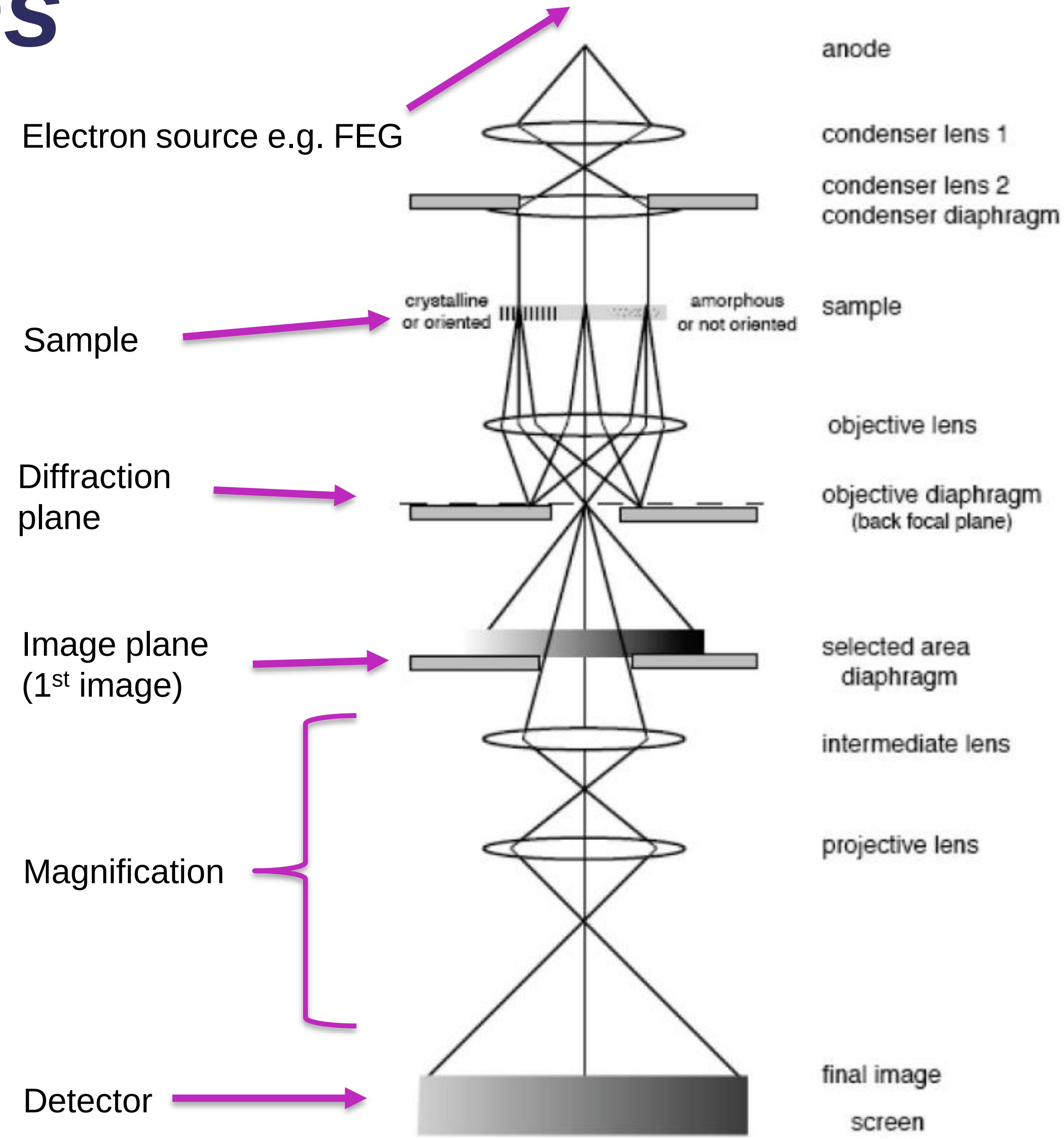
Transmission electron microscopy (TEM)
(cf. scanning electron microscopy SEM)

Thin samples, typically 300 nm or less.

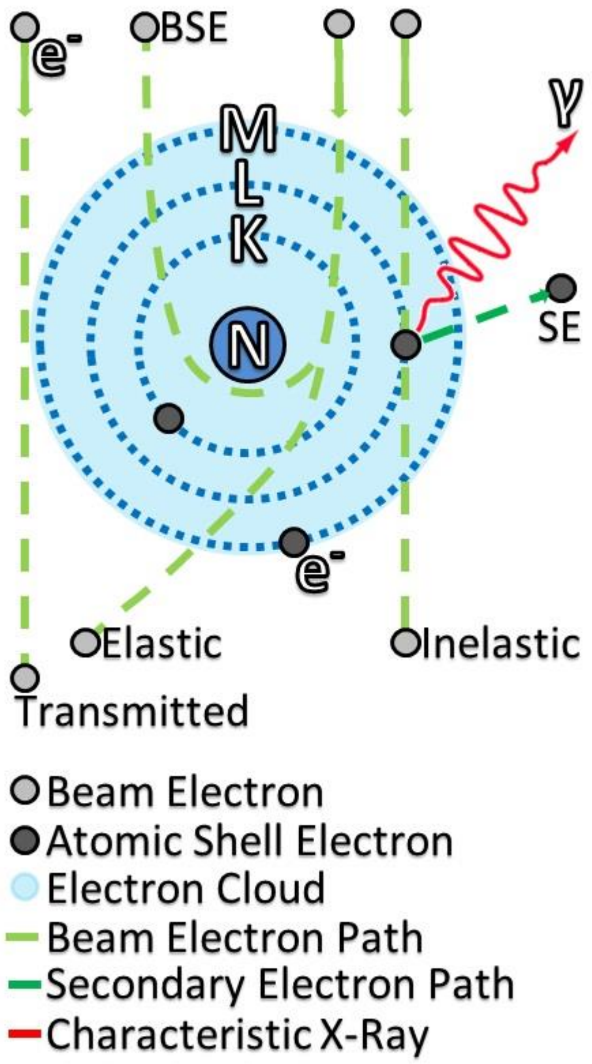
All under vacuum.

Direct electron detectors.

High resolution due to short wavelength.
Titan Krios at 300 keV is 0.018 Å



e^- scattering



Electrons scatter off the electrostatic potential of the sample

+ve from nuclei, -ve from electrons

Elastic scattering – desired signal

Inelastic scattering – noise, radiation damage

Electrons scatter strongly (compared with X-rays or neutrons).

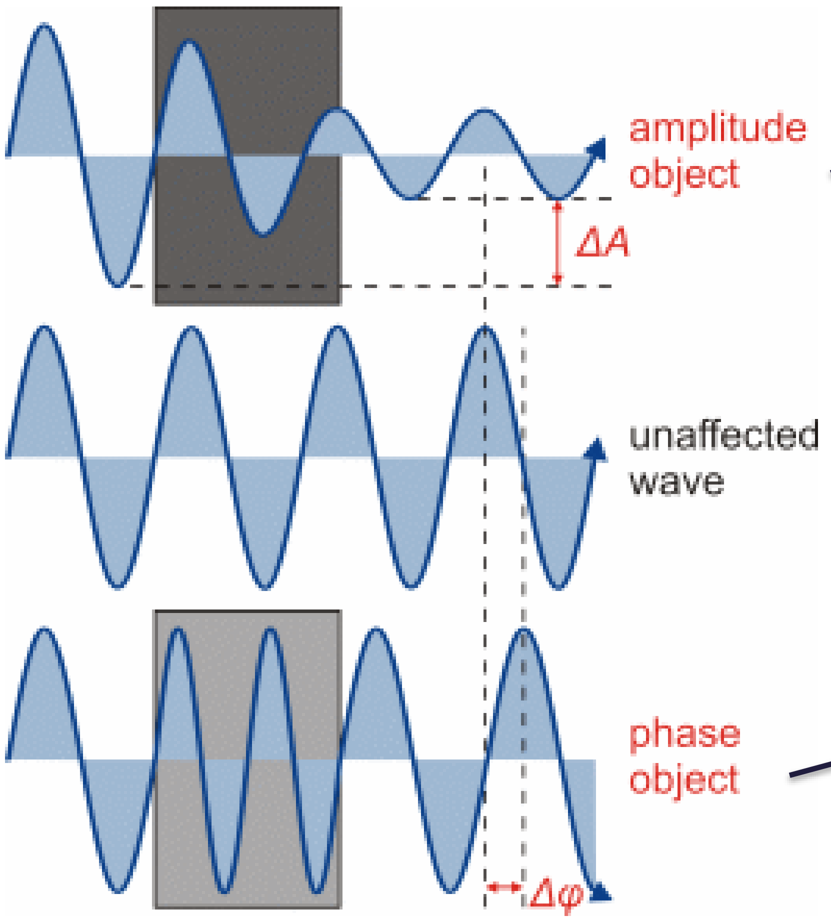
Need thin samples (~< 200 nm).

Issues for thicker samples:

High proportion of inelastic scattering.

Multiple elastic scattering.

Image formation



Electrons transmitted through thin samples produce very little amplitude contrast – can't see anything.
Some exceptions:

- negative stain
- heavy atoms

Different refractive indices of biological components lead to phase shifts.

*Phase shifts are **not** visible in an in-focus and aberration-free image.*

=> Go out of focus or use phase plates.

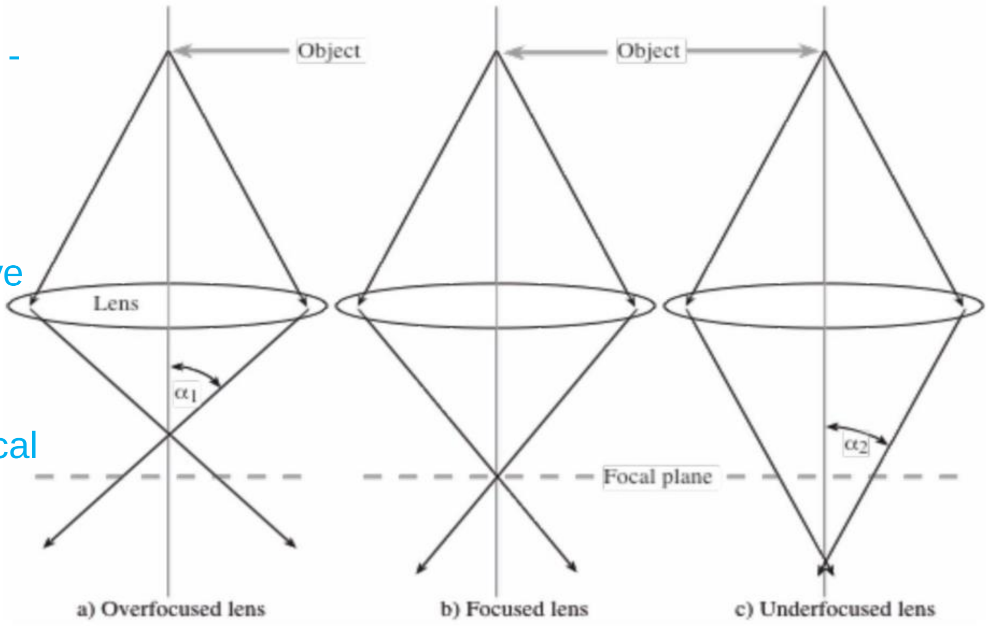
(image taken from: http://home.uni-leipzig.de/pwm/web/?section=introduction&page=phasecontrast)

Defocus

Sample -

Objective lens -

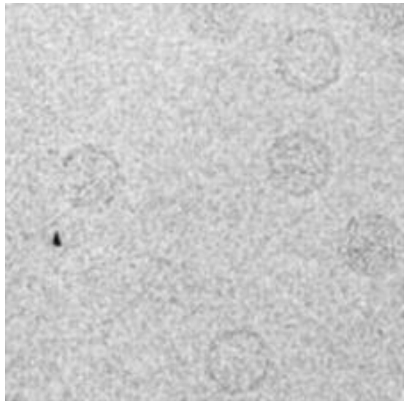
Back focal plane -

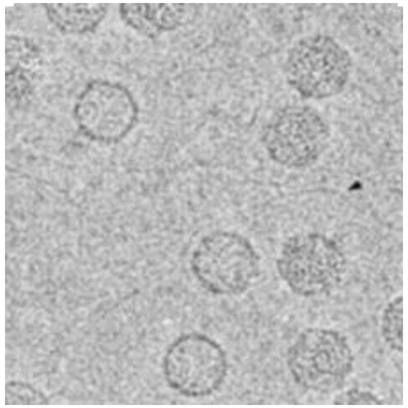


underfocus causes a “delay” in the electrons scattered to higher angles, leading to interferences in the back focal plane that result in enhanced amplitude contrast

Defocus: -0.5 µm -1.0 µm

$f(x)$





Defocus is applied to an image to increase contrast (and find all particles).

Balance against loss of high resolution information.

Contrast transfer function

Weak phase object approximation:

$$X_{ij} = \mathrm{CTF}_{ij} \sum_{l=1}^{L} \mathbf{P}_{jl}^{\phi} V_l + N_{ij}$$

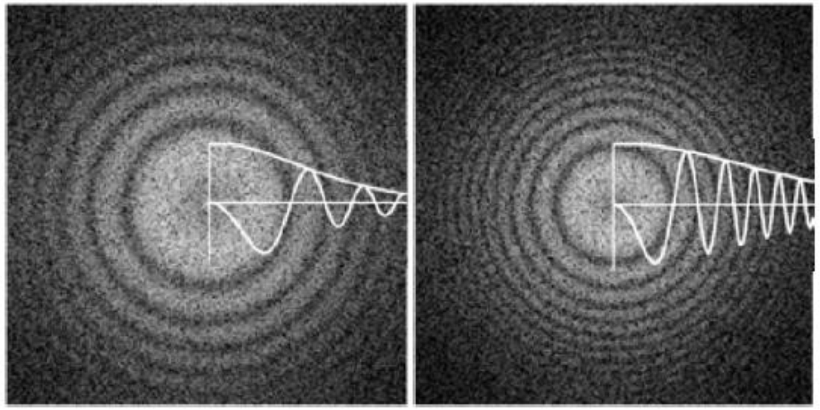
Relates (in Fourier space) 2D images (X) to the projection (P) of the sample volume (V) with added noise (N).

CTF converts simple projection to actual image (as function of frequency) taking into account all the effects of the imaging system.

$$\mathrm{CTF(k)} = -\sin\left[\frac{\pi}{2} C_S \lambda^3 k^4 + \pi \Delta f \lambda k^2\right]$$

λ = wavelength
Cs = spherical aberration
Δf = defocus

No aberration + no defocus => no image !!



CTF visible in power spectrum of 2D images.
Rings (“Thon rings”) are an effect of defocus.

Contrast transfer function

Contrast reversals:

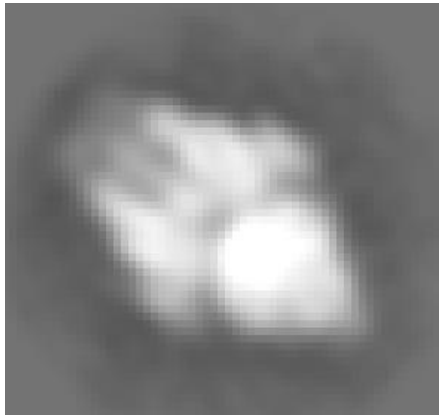
Dark ↔ Light

as function of spatial frequency.

E.g. low frequency features of object are white, high frequency details are black.

Crossings:

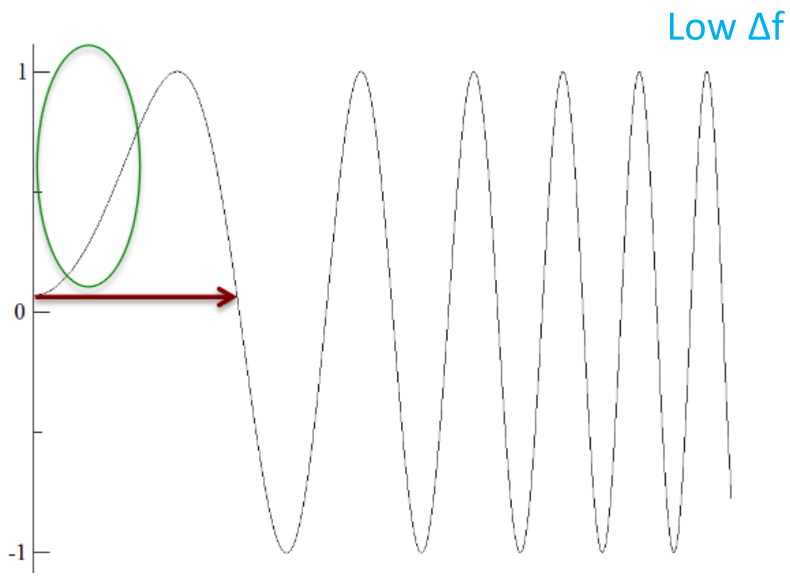
Some frequencies contribute nothing to image (use range of Δf).

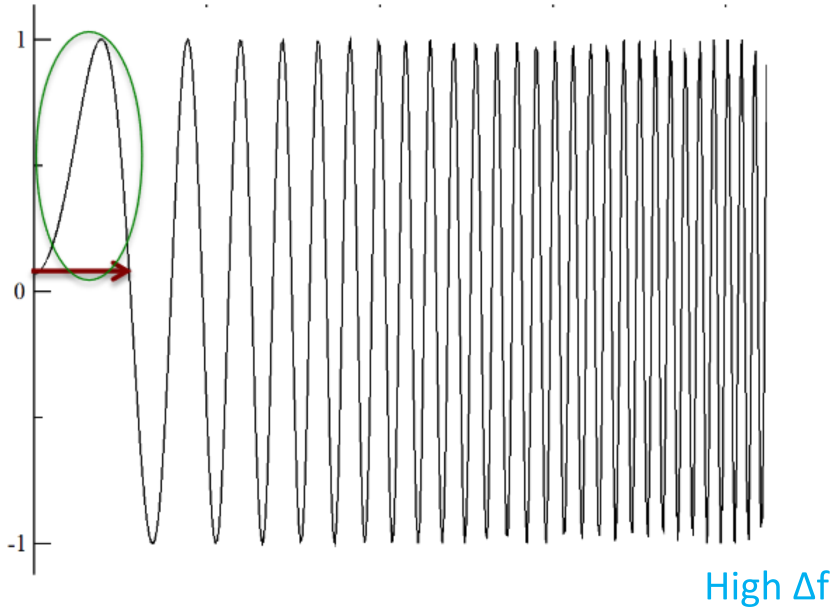


High defocus:

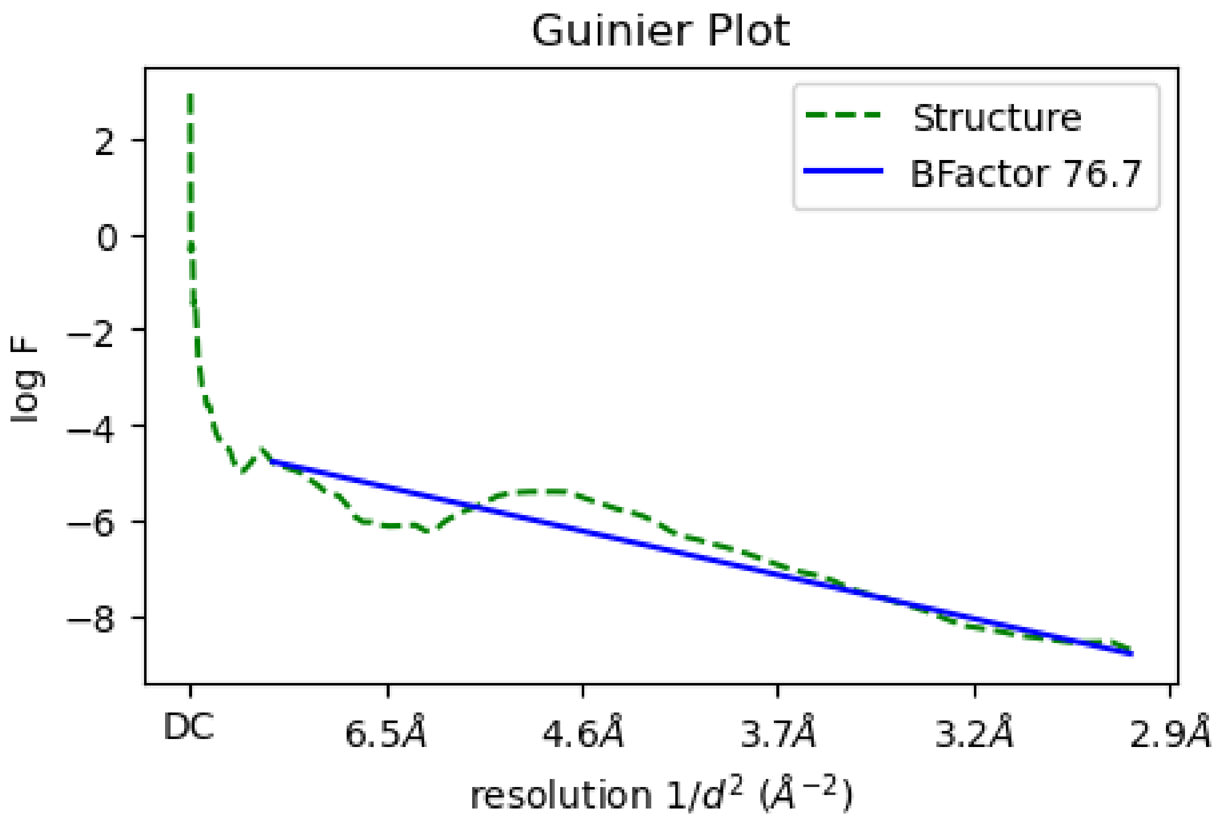
Stronger low frequency signal.

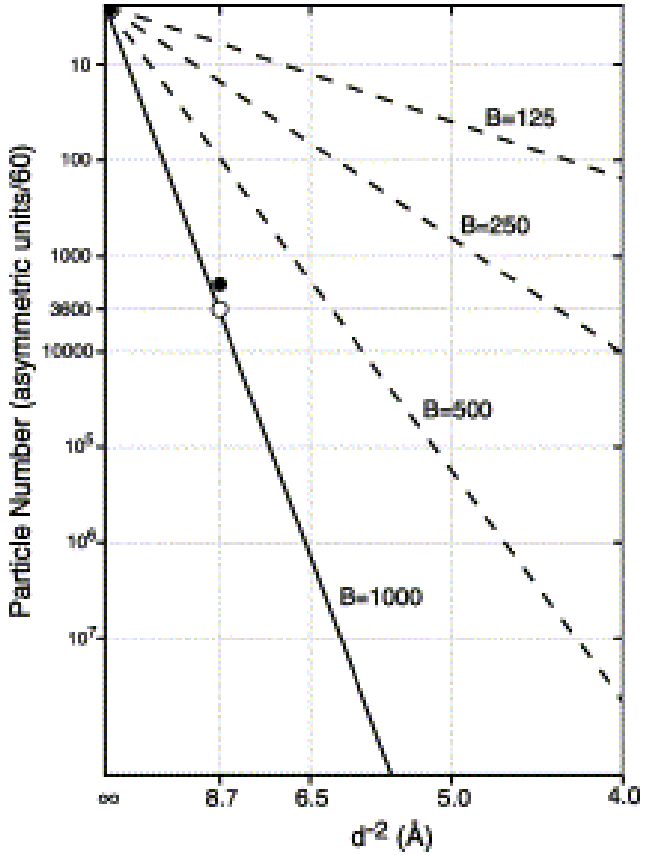
But more crossings.





Amplitude fall-off

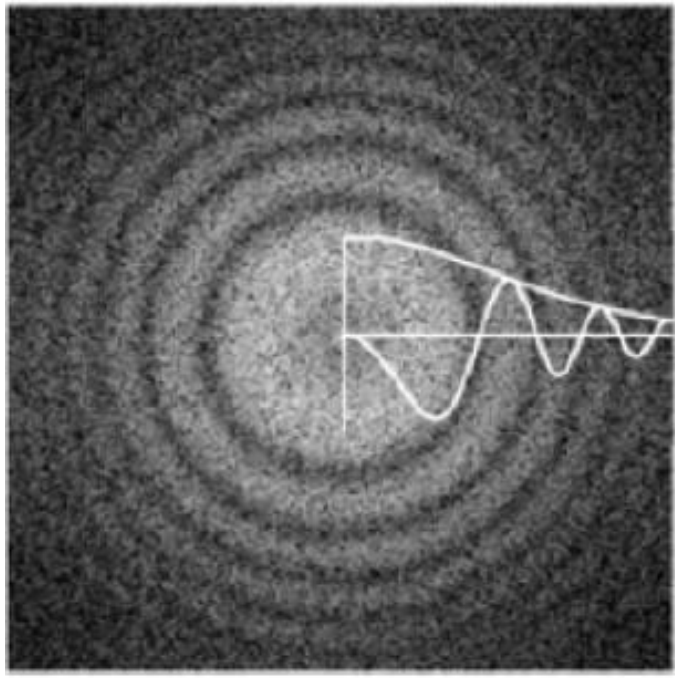




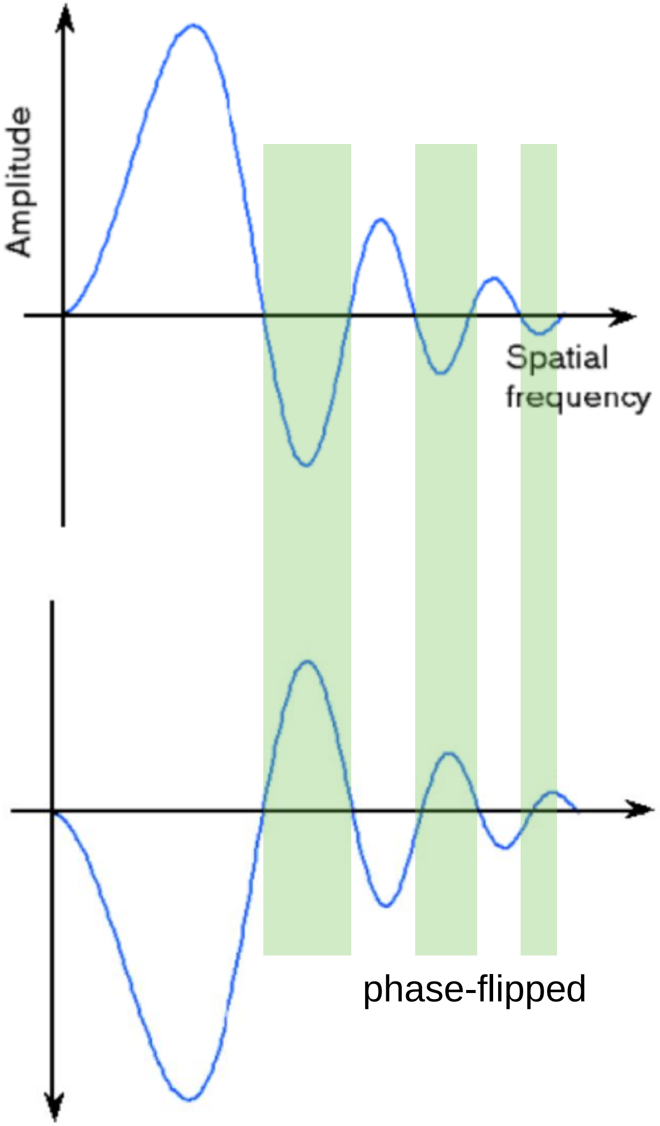
Lower the B factor, the fewer particles needed to reach high resolution.

Cf Wilson plot: log <I> vs resolution

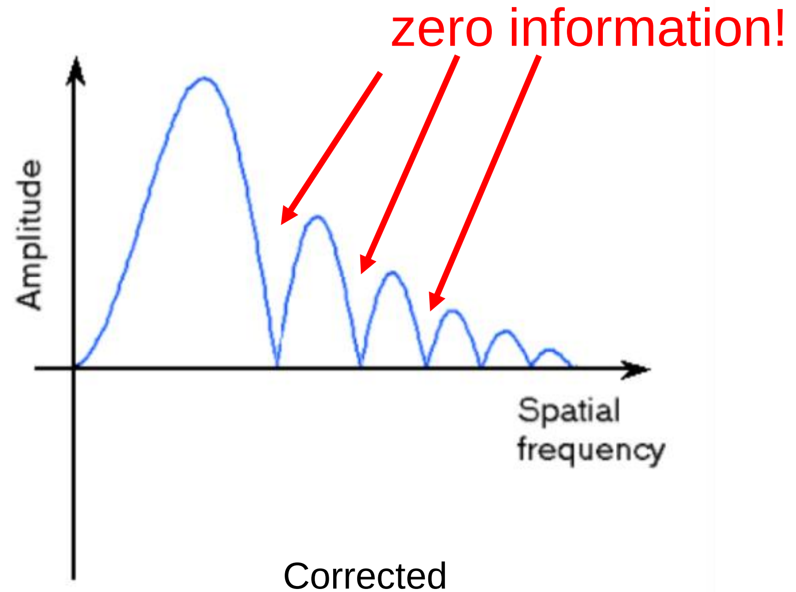
CTF correction



Envelope function
$\exp(-B.k^2/4)$



phase-flipped



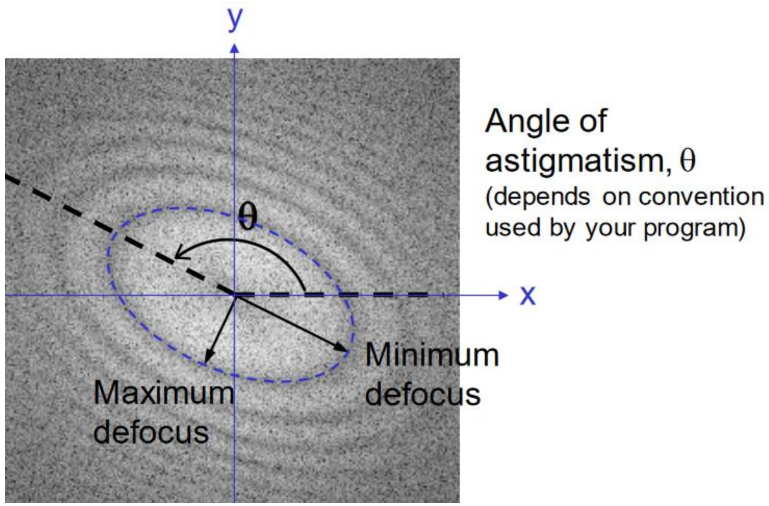
Corrected

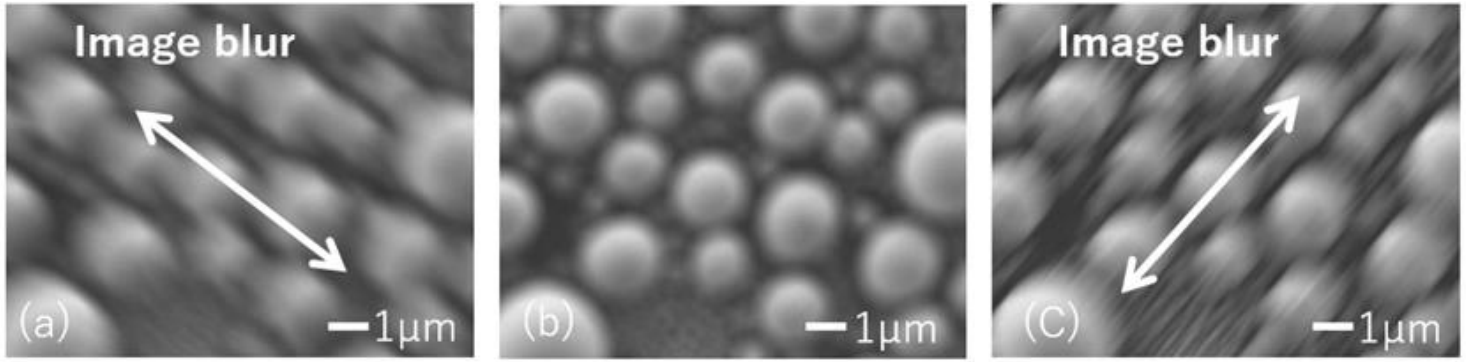
Can choose white particle on black background
or black particle on white background!

Additional Contributions to CTF

Spherical aberration (Cs)
Electrons from the edge of the objective lens are refracted more strongly than those at the center of the lens (defect of magnetic lenses)

Astigmatism
Focus may not be equivalent in all directions

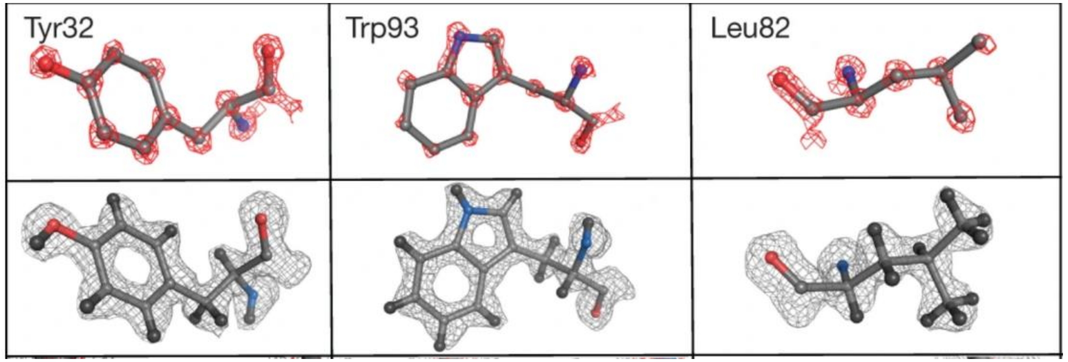


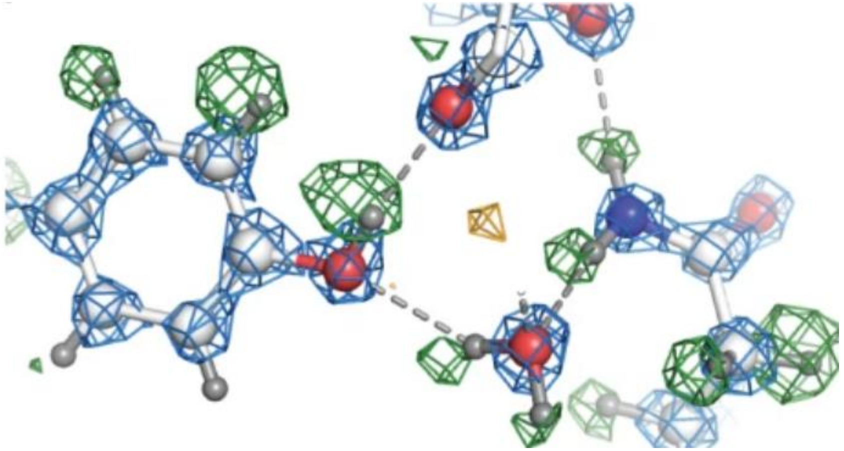


jeol.com

Chromatic aberration

Different wavelengths focus at different planes → try to reduce wavelength spread





Yip et al. Nature (2020)
Apoferritin at 1.25Å (without software corrections)

New microscope with:

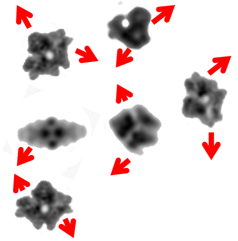
- Monochromator
- Spherical aberration corrector

Nakane et al. Nature (2020)
Apoferritin at 1.22Å

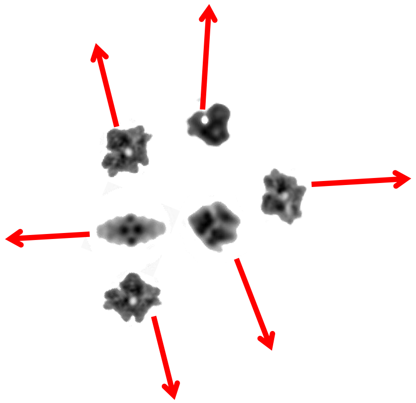
Adapted microscope with:

- Cold field emission electron gun (CFEG)
- Energy filter

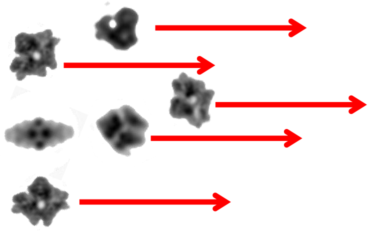
Particle Motion



Stochastic motion
(temperature)



Beam induced motion
(mainly of ice)



Mechanical drift
(microscope stage moving)

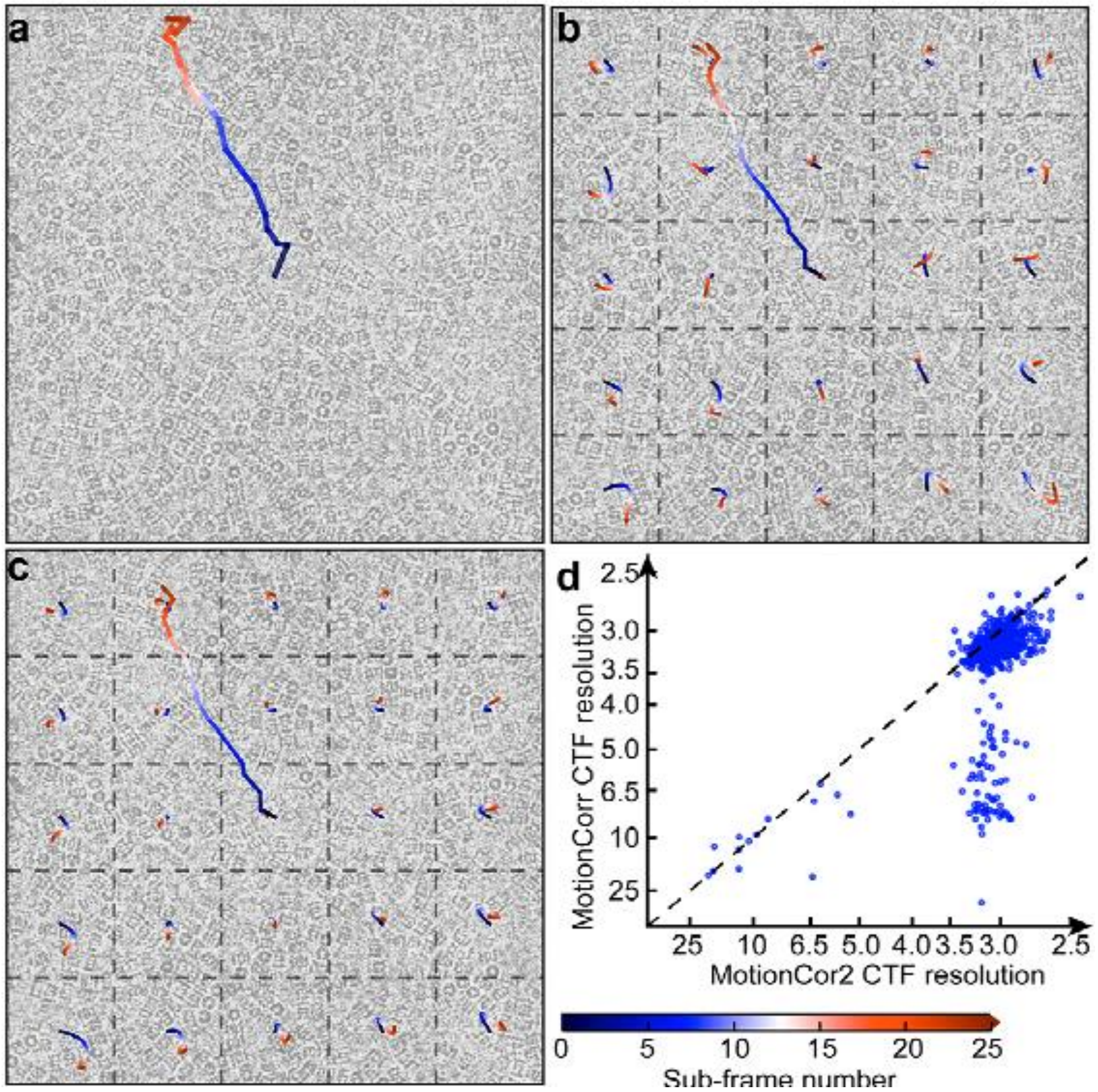
- Motion of the sample during measurement causes blurring.
- Fast frame rate of modern detectors allows movie collection.
- **Collect ~50 sharp movie frames rather than one blurred micrograph**.
- Align and sum.

Micrograph Motion Correction

Align frames of movie to remove blurring.

Can also align patches.

Later, can align individual particles (once known).

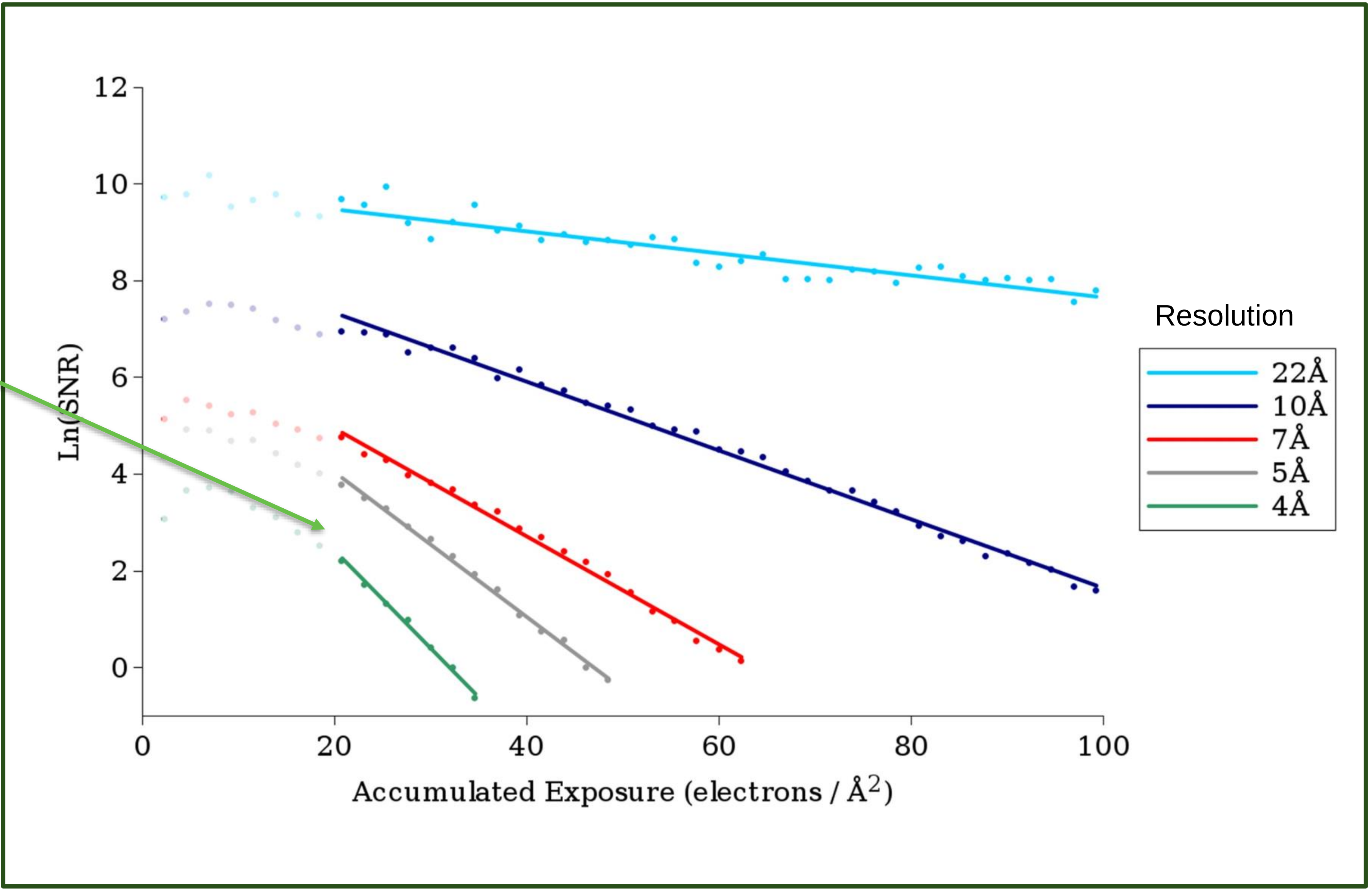


Dose Weighting

Proteins suffer radiation damage from the electron beam

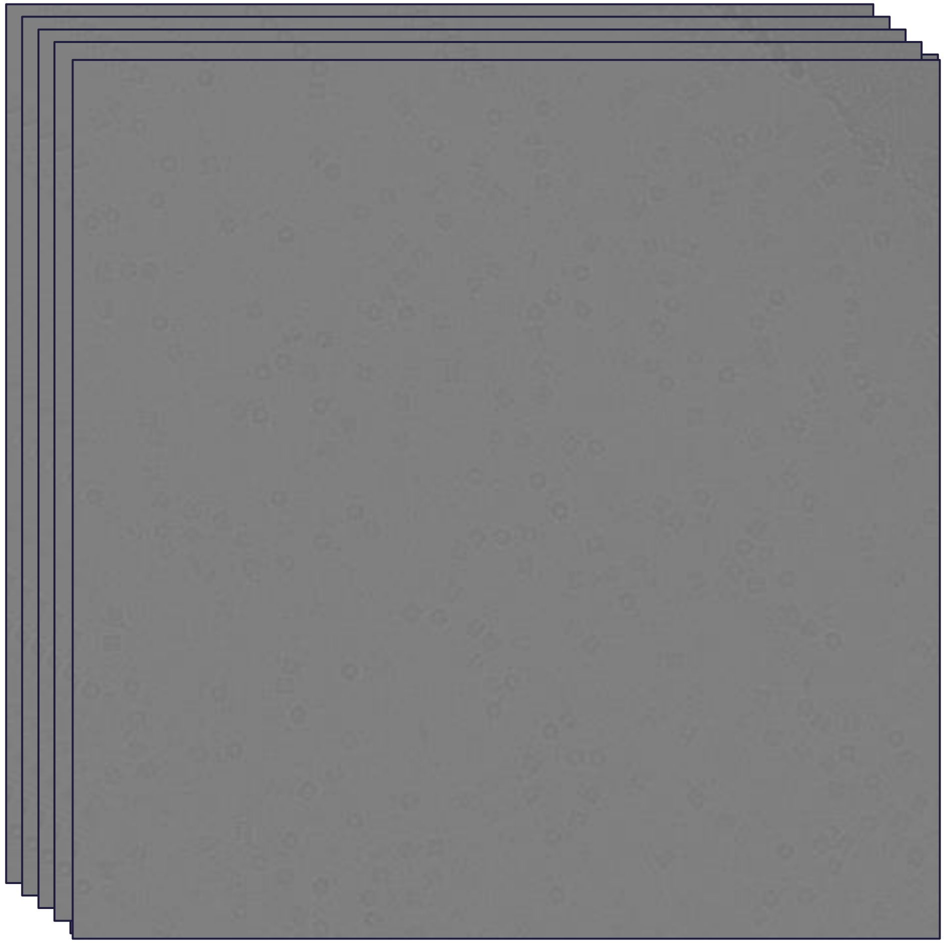
High resolution information is lost first

Dose weighting down weights high resolution information from later frames



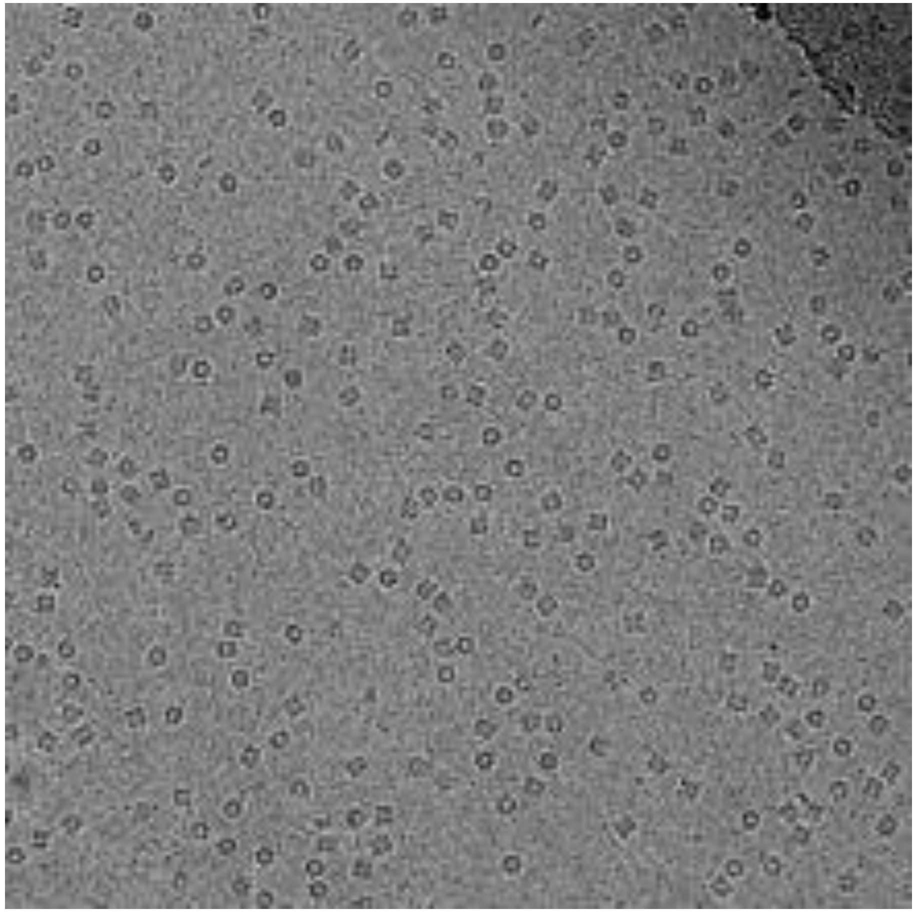
Grant and Grigorieff (2015) Elife 4:e06980.doi: 10.7554/eLife.06980

Movies to Corrected Micrographs



Multiframe move

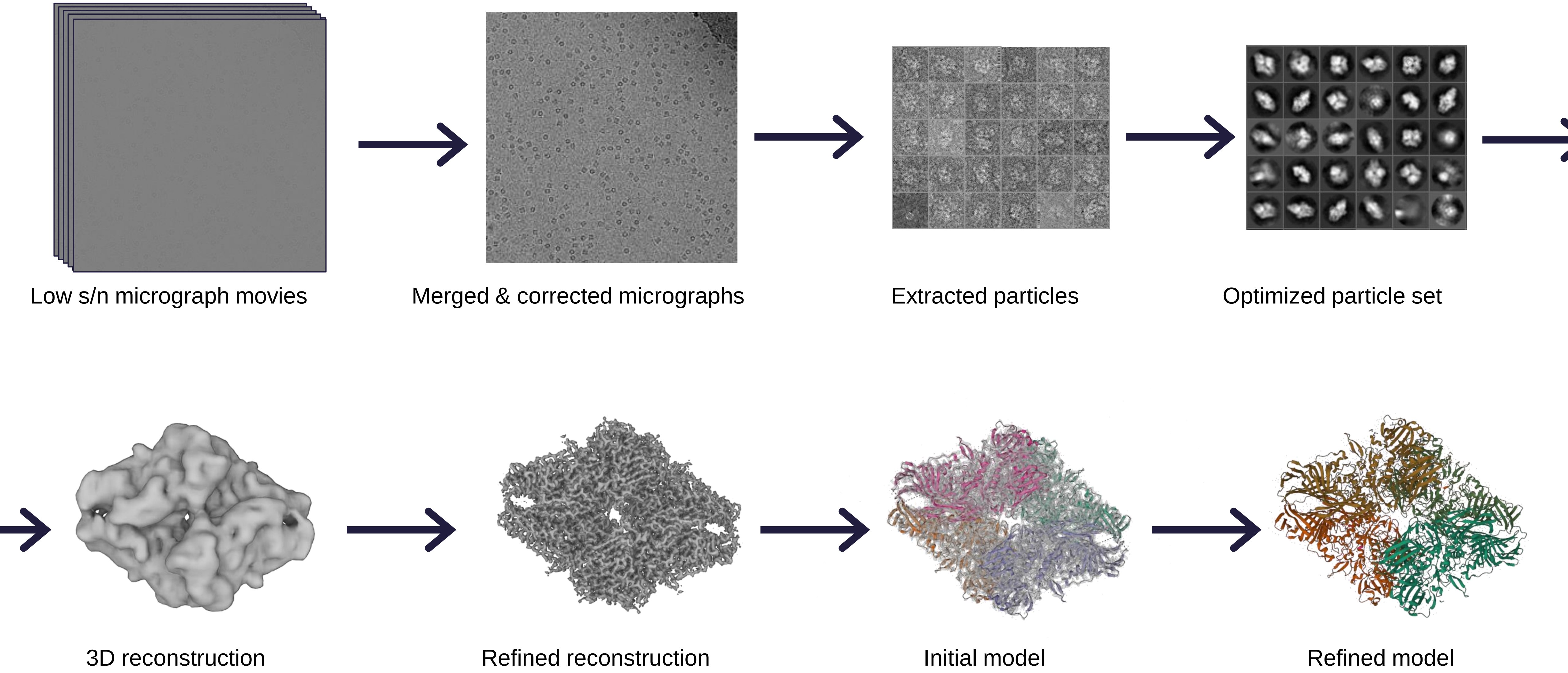
Motion correct
Dose weight
Sum



Corrected micrograph

Correct motion and damage.

CryoEM in one slide

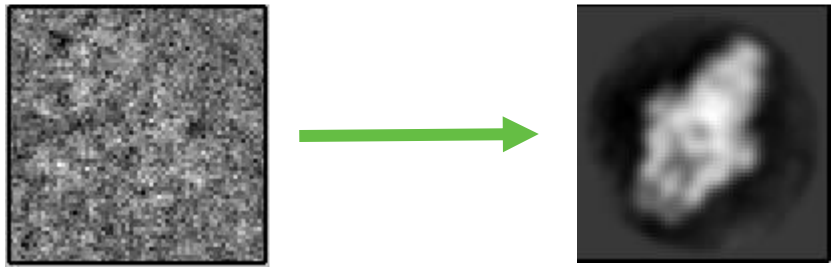


More later …

Science and Technology Facilities Council

Experimental requirements

Sample requirements



Single Particle Analysis:
Average thousands of views of identical particles

Smaller amount of protein than crystallisation assuming:

- well-behaved on grid
- find total number of particles for desired resolution

Classification of particles can allow for digital purification.
BUT still better to have pure, monodisperse, rigid molecule.

Lower size limit:
Theoretically = 42 kDa
Broken through denoising - Blush

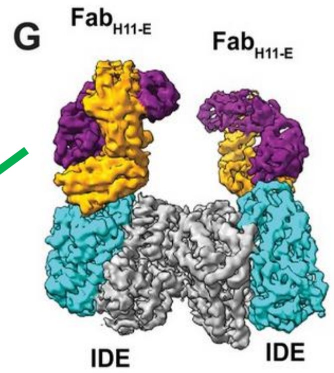
Sample types

Purified complexes
Membrane/vesicle extracts
Viruses/organelles
Cell cultures
Tissues

Improving the sample

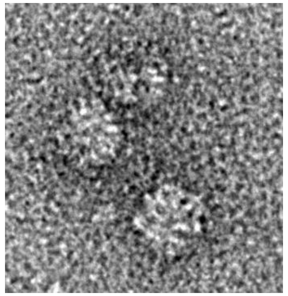
Stabilise through mutation or added components.

Antibodies can help particle alignment.

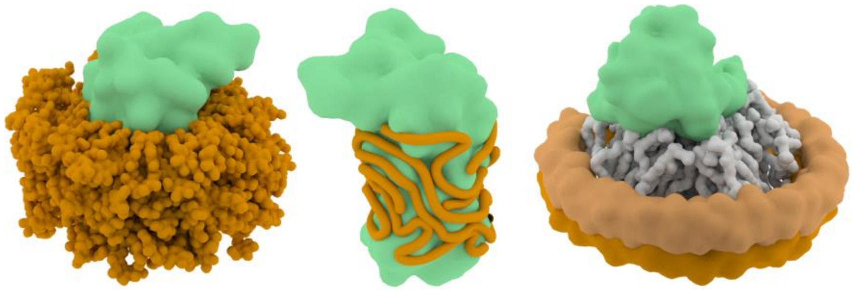


Cross-linking e.g. GraFix method.

Start with negative stain EM.



Membrane proteins in detergent, amphipols, lipid nanodiscs.



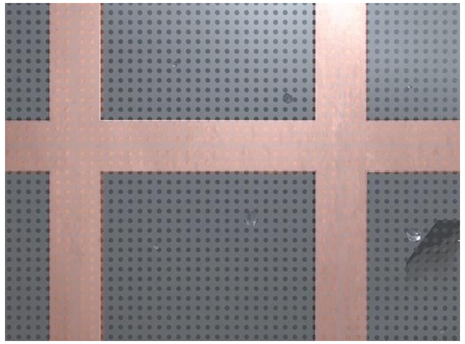
Protein complex stabilisation

Particle	Resolution (Å)	Stabilization method	EMDB Accession No.
β-galactosidase	1.9	Small molecule inhibitor (PETG)	EMD-7770
Vps4	3.2	Non-hydrolyzable nucleotide analog (ADP·BeFx)	EMD-8887
Ribosome Quality Control Complex	8.2	Catalytic inactive mutant	EMD-6170
Insulin degrading enzyme	3.7	Fab antibody fragment	EMD-7062

Membrane proteins

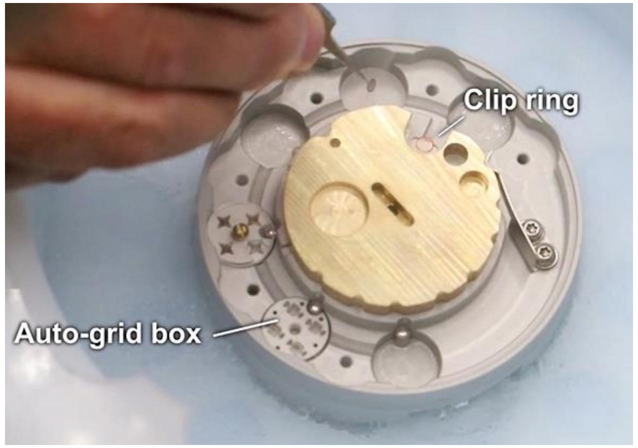
Particle	Resolution (Å)	Stabilization method	EMDB Accession No.
Orco	3.5	Detergent (digitonin)	EMD-7352
TRPV6	4.0	Amphipols	EMD-7121
Kv1.2-2.1	3.3	Nanodisc	EMD-9024

CryoEM experimental workflow



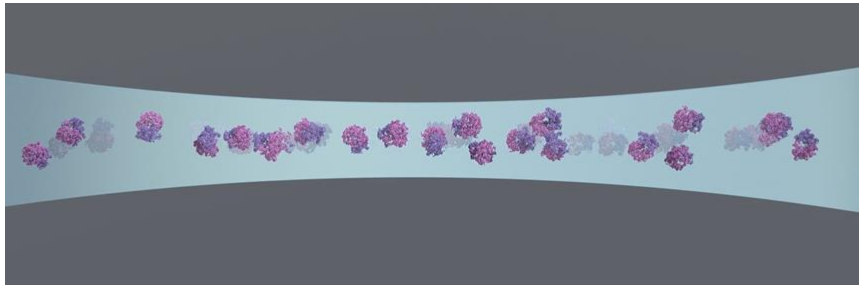
Apply sample to grid.
Metal grid (e.g. Cu, Au).
Support film.

Blotting and vitrification
- Vitrobot
- control temperature, humidity, blot force, and blot time

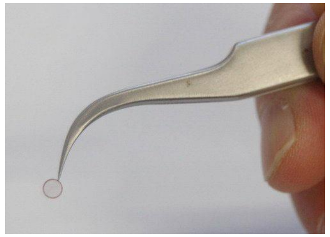


Loading

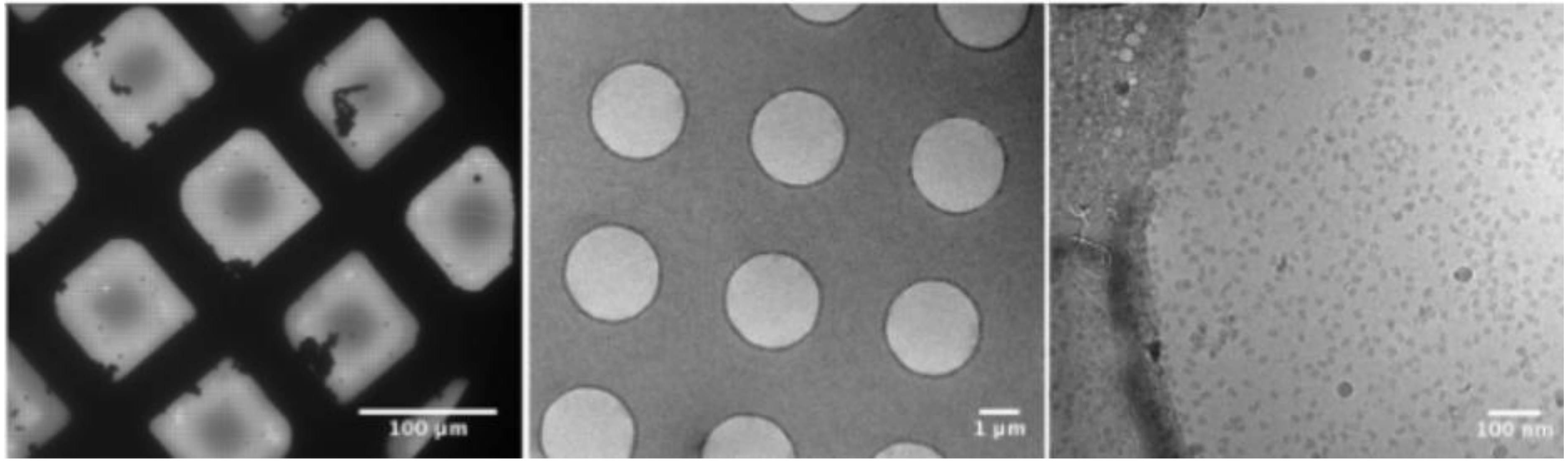
Want <u>even distribution</u> throughout the holes of the support, uniform <u>vitreous</u> ice, broad range of <u>orientations</u>.



Data collection



Grid is large (!) Need to plan where to collect data.
Initial screening: images recorded at range of magnifications to check ice and particle quality.

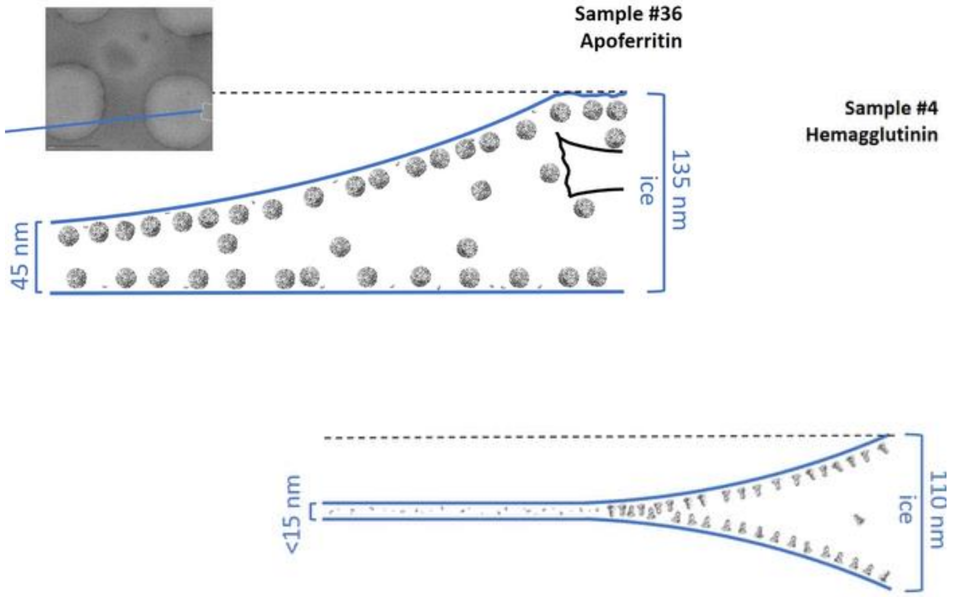


Grid view: Low magnification views to check general ice quality. Thick ice may block or obscure views of grid squares. Vitrified grids may show a gradient of ice thickness.

Square view: Midrange magnification to confirm ice quality within grid squares. May reveal presence of vitreous or crystalline ice, contamination, and variations in ice thickness.

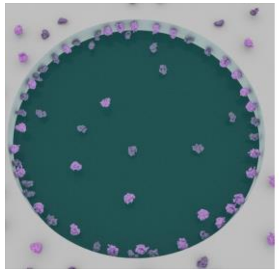
Hole view: High magnification views to evaluate particles. Check if distribution of mono-disperse particles throughout the hole. Small particles may only be visible at high defocus levels.

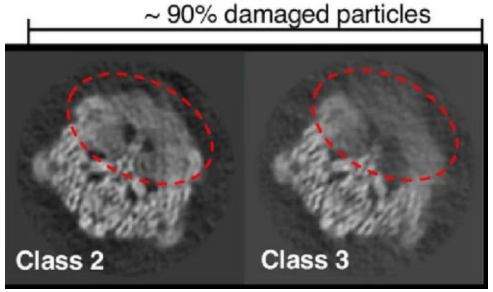
Practical considerations



Problems

- Particles fail to enter holes, stick to support film.
- Preferred orientation: limited available views.
- Association with air-water interface.
- Denaturing or aggregation.
- Complexes fall apart.



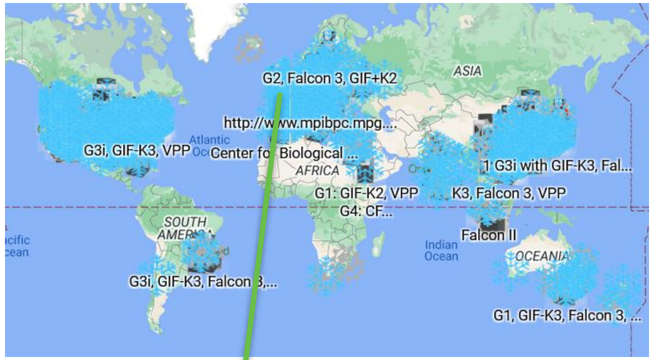


Noble et al eLife (2018)
“we performed fiducial-less tomography on over 50 different cryoEM grid/sample preparations to determine the particle distribution within the ice and the overall geometry of the ice in grid holes”

Solutions (?)

- Mild detergent
- Solid support over holes (e.g. amorphous carbon or graphene)
- Tilt grid

Microscope time



- electron Bio-Imaging Centre (eBIC) at Diamond Light Source, UK.
- 24/7 **free** user access through peer-review process.
- 5x Titan Krios microscopes, a Talos Arctica, two Glacios, a Scios and an Aquilos cryo-FIB/SEM, and a Leica CryoCLEM.

Synchrotron-like access: (1) rapid access, (2) block allocation group, (3) industrial access.
Acceptance criteria:

- Scientific excellence.
- Evidence of sample suitability for high-resolution data collection (e.g. particle distribution in ice, 2D class averages).
- Expected to do grid screening on low-end microscope.

Typically 48 hour session, 4 – 11 grids.
May need to combine data from separate sessions.

Technology advancements

Tundra from TFS, operating at 100 kV

Cheaper to buy, cheaper to run.
Smaller footprint.
Resolution of apoferritin 2.1 Å

Detectors appropriate to 100kV required (under development)

Improved grid preparation

Spotiton.

Piezoelectric inkjet dispensing of drops onto passing grid.
Avoid blotting step.

Chameleon.

Based on Spotiton.
Self-wicking grids.

Training resources

Online resources

LMB Electron Cryo-microscopy Course 2023
https://www2.mrc-lmb.cam.ac.uk/research/scientific-training/electron-microscopy/

CryoEM 101 and CryoET 101
https://cryoem101.org

Grant Jensen cryoEM course
https://cryo-em-course.caltech.edu/

Mailing lists

CCP-EM: https://www.jiscmail.ac.uk/CCPEM

3dem: http://3dem.ucsd.edu/mailinglist.shtm

Courses

CCP-EM Icknield workshops
Birkbeck EMBO course

2024 Spring Symposium X

https://www.ccpem.ac.uk/training/spring_symposium_2024/spring_symposium_2024.php

CCP-EM core team

CCP4 core team

STFC Business & Innovation

CCP-EM Commercial License Holders

CCP-EM Collaborators

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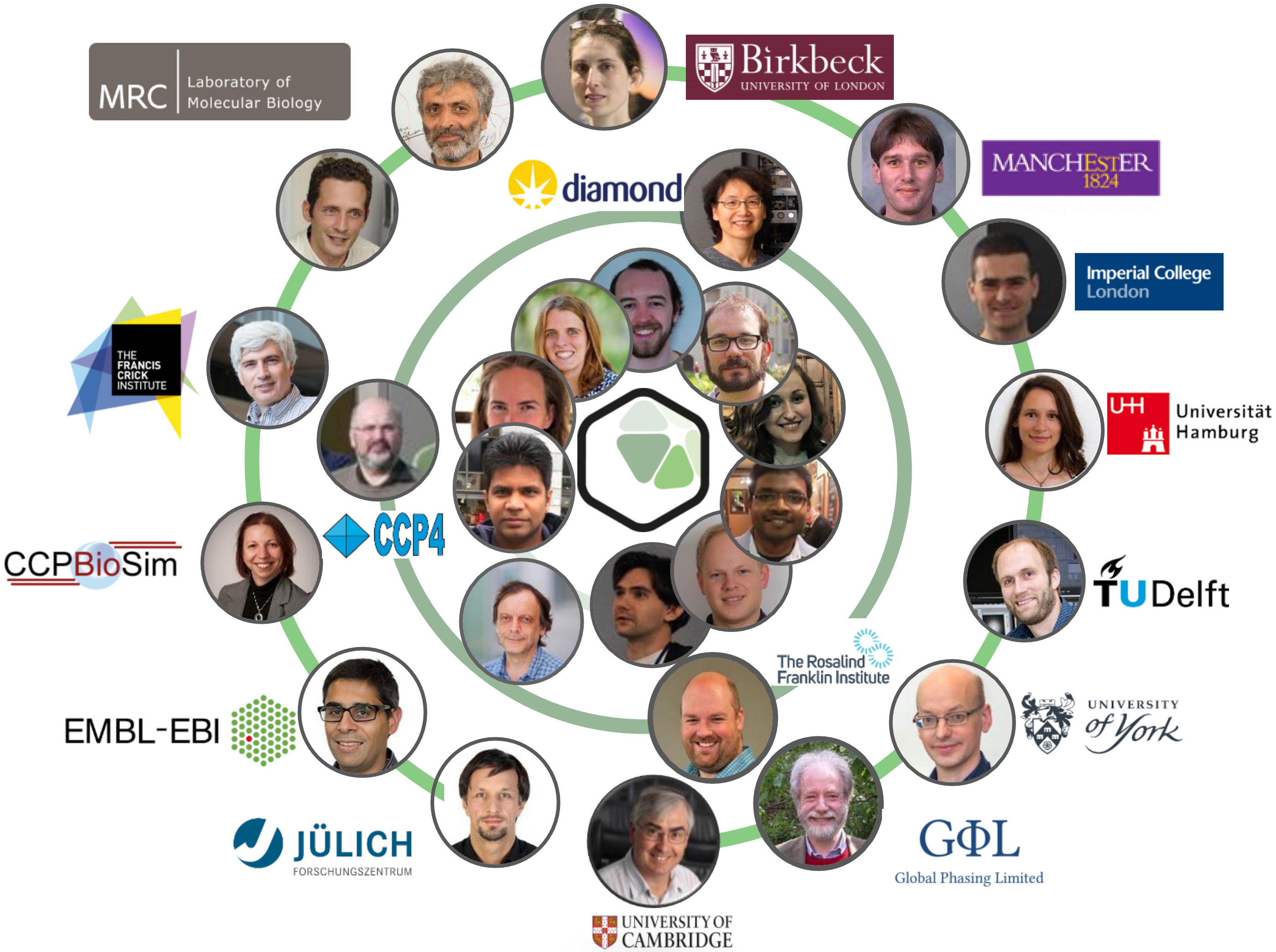
Tom Burnley



CCP-EM

Research Complex at Harwell

UKRI

Medical Research Council

wellcome

Department for Business, Energy & Industrial Strategy

GΦL Global Phasing Limited